AD_____

Award Number: W81XWH-04-1-0263

TITLE: Sonic Hedgehog Signaling Promotes Tumor Growth

PRINCIPAL INVESTIGATOR: Wade Bushman, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Wisconsin
                                               Madison, WI 53792

REPORT DATE: February 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
                          Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE		*Form Approved* *OMB No. 0704-0188*
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)* 01-02-2006	2. REPORT TYPE Annual	3. DATES COVERED *(From - To)* 26 Jan 2005 - 25 Jan 2006
4. TITLE AND SUBTITLE Sonic Hedgehog Signaling Promotes Tumor Growth		**5a. CONTRACT NUMBER**
		5b. GRANT NUMBER W81XWH-04-1-0263
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) Wade Bushman, M.D., Ph.D. E-Mail: bushman@surgery.wisc.edu		**5d. PROJECT NUMBER**
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Wisconsin Madison, WI 53792		**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		**10. SPONSOR/MONITOR'S ACRONYM(S)**
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT:
   We have shown that the *Sonic hedgehog (Shh)* signaling pathway is absolutely required for normal prostate development and our studies performed with the support of the DOD New Investigator award indicate that *Shh* signaling promotes tumor growth. This proposal addresses **the hypothesis that Sonic hedgehog signaling promotes tumor growth by activating stromal cell gene expression.** To address this hypothesis, we have developed the DLPlacZ-LNCaP xenograft, a model that provides us with the opportunity to selectively assay gene expression in the stromal and epithelial compartments of the tumor using species specific PCR primers and to make specific modifications in stromal cell gene expression. We will use this model to: (1) determine whether *Shh* promotes tumor growth by activating expression of *Gli-1* in the tumor stromal cells; (2) characterize the mechanisms by which tumor growth is promoted; and (3) examine the action of a specific stromal *Shh* target genes in tumor growth.

15. SUBJECT TERMS
LNCaP, Prostate Cancer, Xenograft, Stroma, Sonic Hedgehog

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	61	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

INTRODUCTION:

To address the hypothesis that Sonic hedgehog signaling promotes tumor growth by activating stromal cell gene expression, we planned to use a xenograft tumor model in which LNCaP cells are co-injected with cloned, immortalized lacZ expressing stromal cells. The value of this tumor model is that it would provides us with the opportunity to selectively assay gene expression in the stromal and epithelial compartments of the tumor using species specific PCR primers and to make specific modifications in stromal cell gene expression. We planned to use this model to: (1) determine whether Shh promotes tumor growth by activating expression of Gli1 in tumor stromal cells; (2) characterize the mechanisms by which tumor growth is promoted; and (3) examine the action of specific stromal Shh target genes in tumor growth.

BODY:

Studies performed before this proposal was funded compared xenograft tumors made with co-injection of stromal cells from the urogenital sinus or from the different lobes of the adult mouse prostate. Based on those studies, we elected to use a mesenchymal cell line in the experiments proposed. A paper describing the isolation and characterization of the UGSM-2 cell line has been accepted for publication in The Prostate. A copy of that paper is attached.

As proposed in Specific Aim1, we have characterized the growth and androgen dependence of the LNCaP-UGSM2 xenograft and shown that Shh overexpression increases growth of the xenograft tumor (Figure 1).
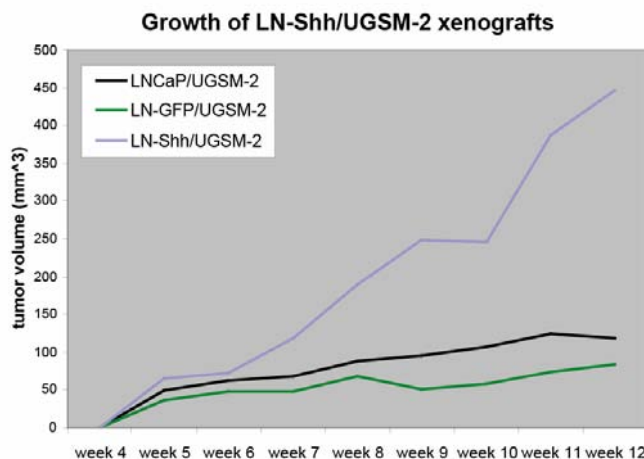

Figure 1. Shh-induced growth of LNCaP-UGSM2 tumors.

To verify that paracrine Hh signaling underlies the Shh growth effect, we analyzed the ability of Shh to induce growth of parent LNCaP in tumors composed of an equal mixture of parent LNCaP and LN-Shh cells. The tumors composed of a mixture of parent LNCaP and LN-Shh cells grew at a faster rate than parent LNCaP tumors, validating the Shh effect in these tumors. Surprisingly, tumors generated by injecting a mixture of LNCaP and LN-Shh cells were composed primarily of parent LNCaP cells after 8 weeks of growth in vivo, confirming that Shh does not have a specific effect on LN-Shh cells, but rather induces paracrine factors that induce growth of parent LNCaP (Figure 2).
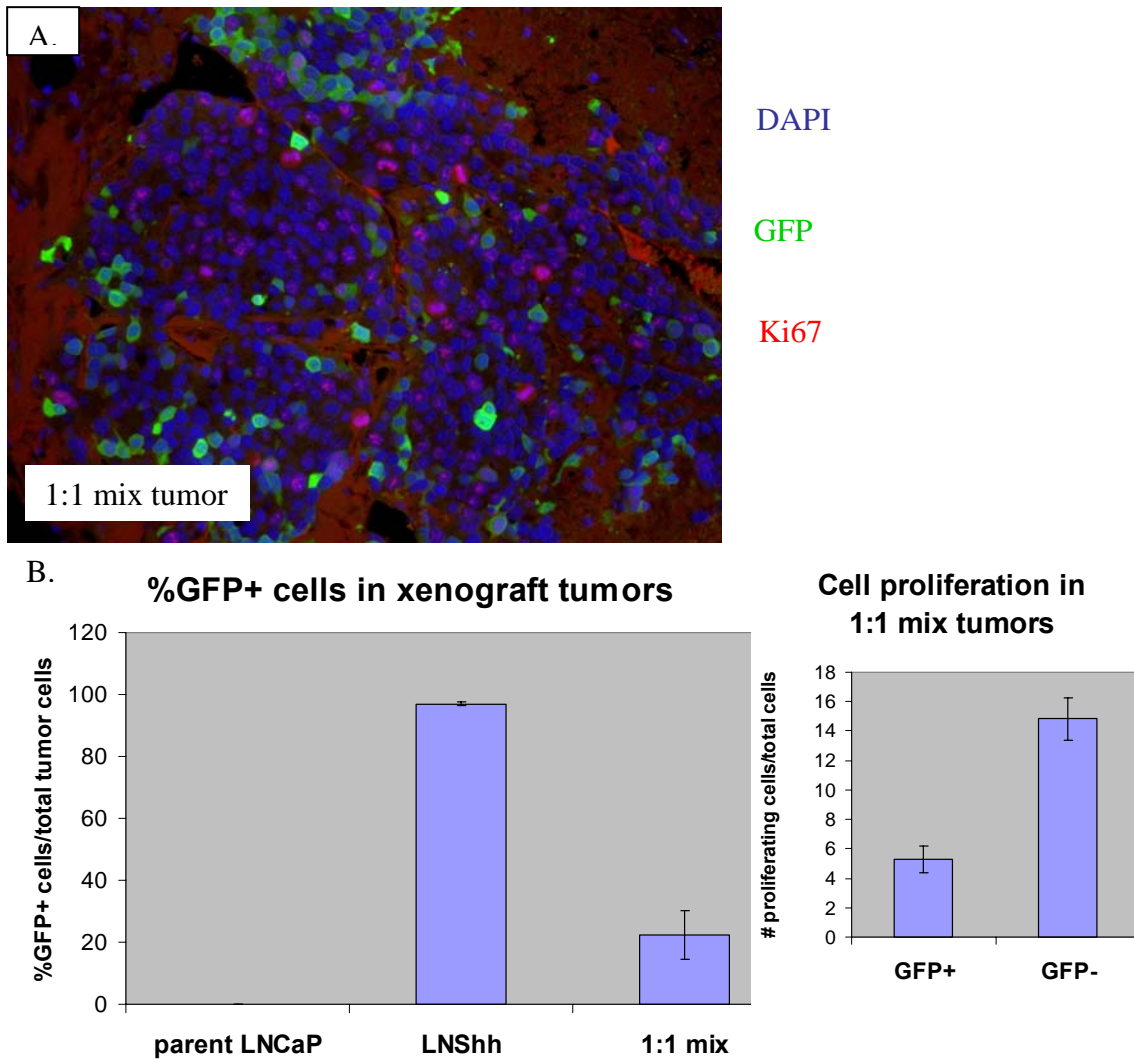
Figure 2. Shh signaling induces paracrine signaling that stimulates growth of LNCaP. (A) immunohistochemical staining for GFP and Ki67 shows that parent LNCaP are the main component of LNCaP + LN-Shh-GFP 1:1 mix tumors. (B) Enumeration of GFP+ LN-Shh and GFP- parent LNCaP in tumors and corresponding proliferation rates for each in 1:1 mix tumors. Tumors are composed mainly of parent LNCaP, supporting that Shh does not preferentially induce proliferation of cells that secrete it and that the Shh growth effect occurs by activating Hh signaling in cells other than LN-Shh.

To determine if stromal Hh signaling activation mimics the Shh growth effect, we generated a stromal cell line that lacks Gli3, a repressor of Hh signaling. Gli3 null stromal cells exhibit active Hh signaling in the absence of Shh. Co-injection of Gli3 null stromal cells resulted in rapid growth of tumors when compared with tumors containing wild-type stromal cells (Figure 3).
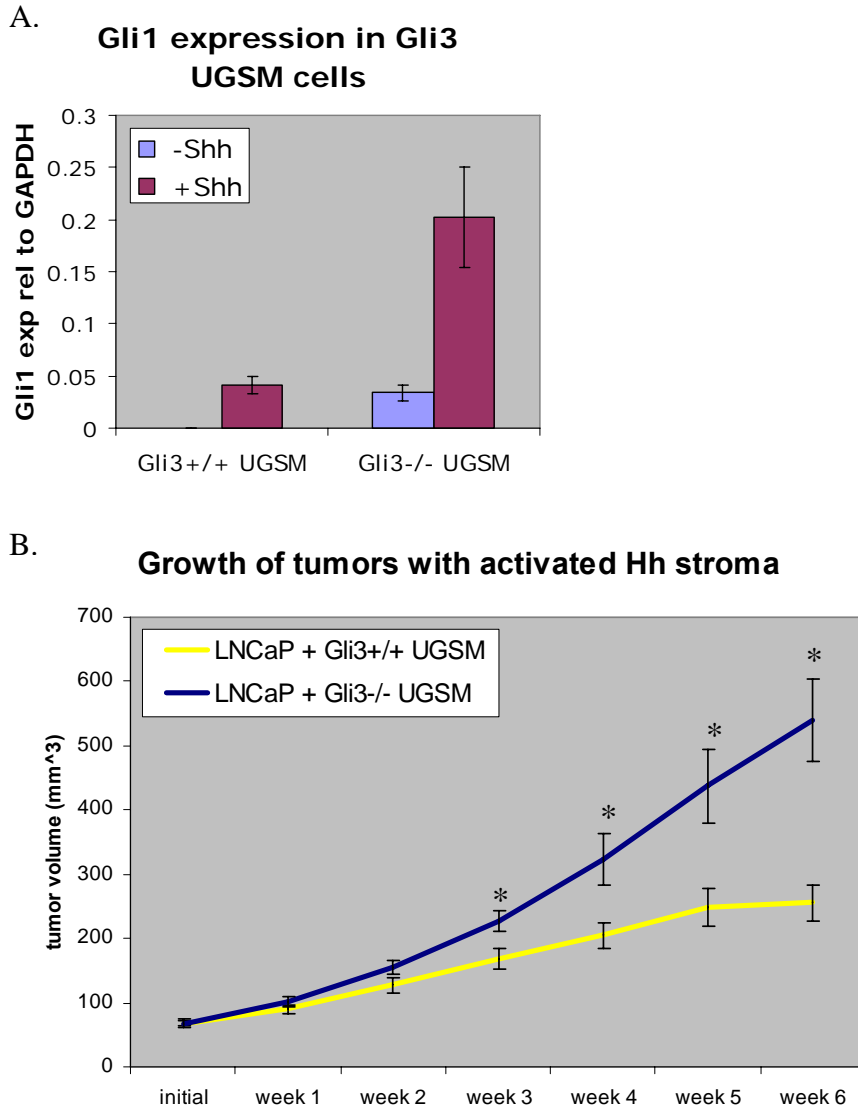
A.

Gli1 expression in Gli3 UGSM cells

B.

Growth of tumors with activated Hh stroma

Figure 3. Stromal Hh signaling induces rapid tumor growth. (A) Gli3-/- cells have activated Hh signaling in the absence of Hh ligand. (B) Tumors containing Gli3-/- stromal cells grow faster than tumors containing wild-type stromal cells.

Detailed analysis of Hh signaling in LNCaP revealed that LNCaP do not respond to Shh by increasing expression of the canonical Hh signaling mediators Gli1 and Ptc1 (Figure 4). In fact, expression of Smo in 22RV1 or PC-3 cells does not induce pathway activation as it does in other cell lines (Figure 5). However, expression of Gli1 or Gli2 in 22RV1 or PC-3 cells does induce transcription of Hh target genes Gli1 and Ptc (Figure 6). These studies revealed that intracellular Hh signal transduction in LNCaP is functionally impaired and pathway target genes can only be induced by expression of the final mediators of the Hh transcriptional response. We have recently found that Hh signaling is similarly impaired in LNCaP (not shown), lending credence to the idea that LNCaP are not capable of Hh signal transduction and growth effects on tumors must be mediated by paracrine interactions with other cells in the tumor.
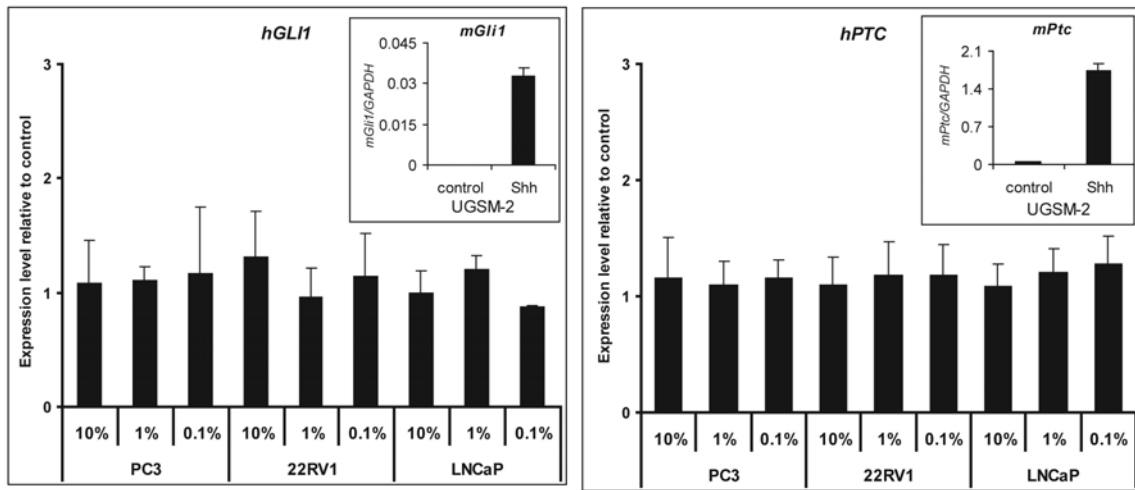
Figure 4. Treatment of Prostate cancer cell lines
with 1 nM purified Shh peptide does not induce expression of Hh target genes Ptc and Gli1. (inset)
Treatment of UGSM-2 mesenchymal cells with the same dose of Shh causes a ~100-fold increase in
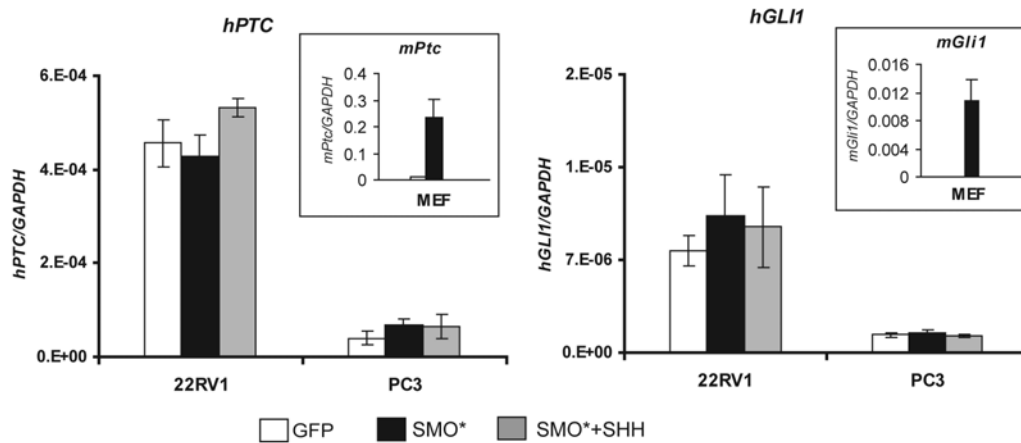expression of Hh target genes Ptc and Gli1.

Figure 5. Expression of constitutively active Smo, an inducer of Hh signaling, fails to induce expression
of Hh target genes in 22RV1 and PC-3 human prostate cancer cell lines, but the same expression construct
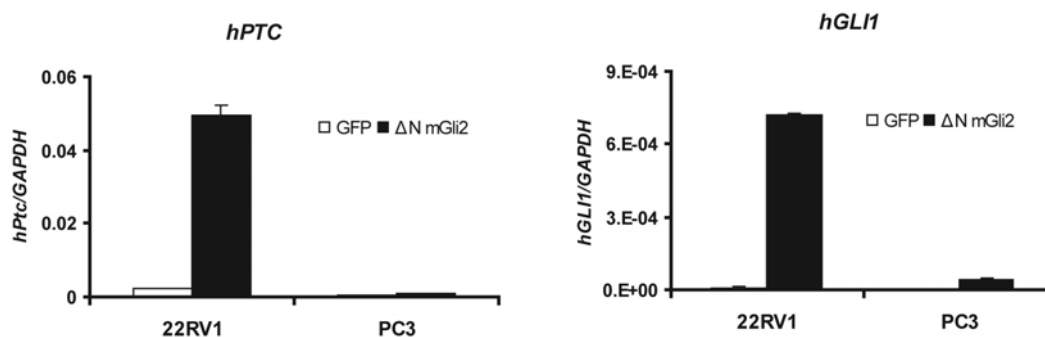faithfully induces Hh signaling in mouse embryonic fibroblasts (inset).

Figure 6. Expression of constitutively active Gli2 in 22RV1 or PC-3 cells induces Hh signaling.

Collectively, these results indicate that Shh expression by LNCaP in LN-Shh tumors
induces stromal Hh signaling that leads to accelerated tumor growth.

As proposed in Specific Aim2, we have identified new Hh target genes in stromal cells and have begun correlating expression of these genes with accelerated tumor growth in LN-Shh tumors. We have identified 40 new target genes and are currently in the process of determining if these genes are modified in LN-Shh tumors with accelerated growth. On the basis of its known role in prostate development, we analyzed expression of Noggin in LN-Shh vs. parent LNCaP tumors. Noggin expression is significantly elevated in LN-Shh tumor stroma (Figure 7).
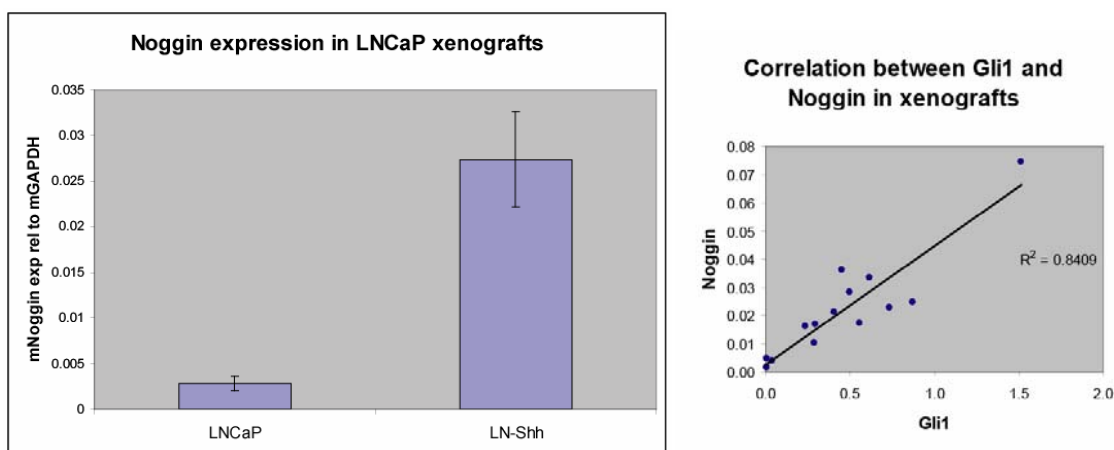
Figure 7. Noggin expression is increased in stroma of LN-Shh tumors and correlates with stromal Gli1 expression.

Noggin is a secreted BMP antagonist and blocks BMP inhibition of LNCaP proliferation in vitro (Figure 8). BMP signaling correlates with expression of Id-1 in LNCaP and LN-Shh tumors exhibit decreased BMP signaling as a result of Noggin overrexpression in the stroma (Figure 9).

Figure 8. Growth of LNCaP in culture was measured daily after treatment with recombinant proteins. BMP-2 and BMP-4 inhibited cell growth while Noggin had no effect. However treatment with BMP together with Noggin reversed the BMP inhibitory effect.

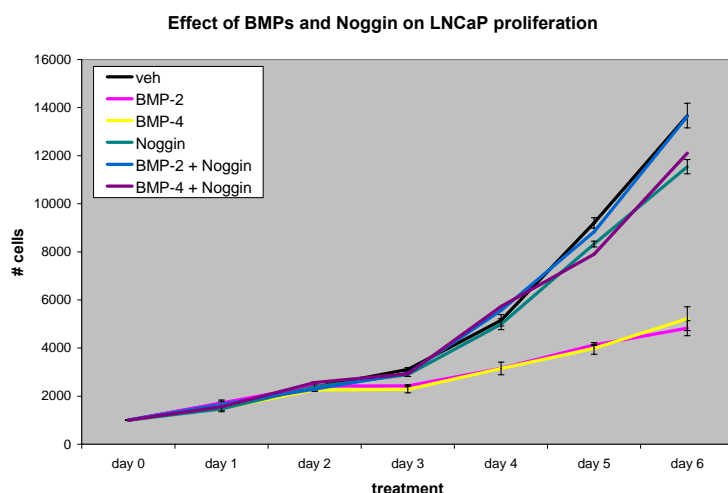**BMP4 induces Id-1 expression in LNCaP**
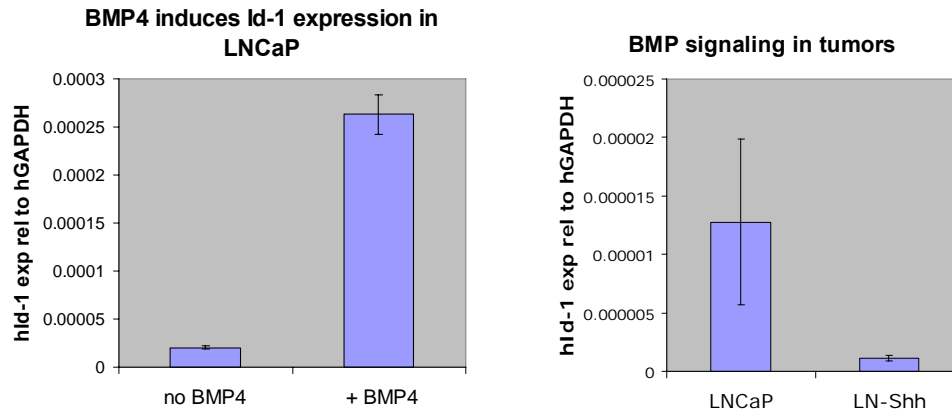
BMP signaling in tumors

Figure 9. Reduced BMP signaling in LN-Shh tumors. BMP4 treatment in vitro induces expression of Id-1 in LNCaP. LN-Shh tumors that express high levels of the BMP antagonist Noggin have reduced BMP signaling, as evidenced by Id-1 expression.

We are currently in the process of identifying other Shh target genes whose expression is modified in LN-Shh tumor stroma. As these genes are identified we will determine appropriate ways to examine the effect of these factors on tumor growth.

To perform the studies described in Specific Aim 3, we have generated UGSM-2 cells which overexpress Smo, Gli1 or Gli2 and UGSM-2 cells which lack Gli1 and Gli2 function. A newly identified dominant negative Gli2 mutant holds promise as a means of repressing Hh signaling in UGSM-2 cells. We have determined that Gli1 and Gli2 overexpression is toxic and lethal to LNCaP, but LNCaP stably overexpressing Smo have been obtained.

The second portion of Specific Aim 3 is to examine the effect of stromal Hh target expression on tumor growth. To this end, we have stably overexpressed Noggin in UGSM-2 stromal cells and generated tumors by combining LNCaP + UGSM2-Noggin cells. Analysis of the growth of these tumors showed that Noggin overexpression in tumor stroma does not accelerate tumor growth, nor is Noggin overexpression additive with Shh for inducing tumor growth (Figure 10). Thus, Noggin does not act alone to stimulate tumor growth.

Growth of tumors with Noggin OE

- LNCaP + UGSM2-Noggin
- LNCaP + UGSM2-GFP
- LNCaP + UGSM2-Noggin + UGSM2-GFP (1:50)
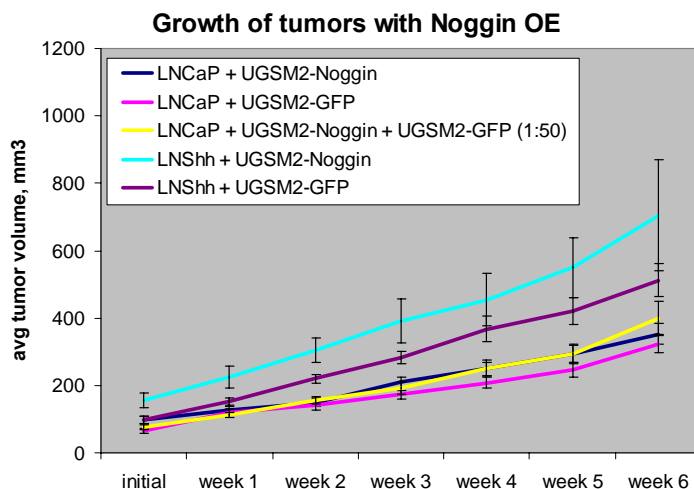- LNShh + UGSM2-Noggin
- LNShh + UGSM2-GFP

Figure 10. Noggin over-expression in tumor stroma does not stimulate tumor growth. Stromal Noggin overexpression does not induce tumor growth in addition to Shh.

9

We are currently in the process of generating stromal cells lines that lack Noggin to examine the requirement for stromal Noggin in the Shh growth effect. As new Shh target genes are identified in Specific Aim 2, we will examine the contribution of these genes to Shh-induced tumor growth.

KEY RESEARCH ACCOMPLISHMENTS:

We have developed and characterized a new mesenchymal cell line that will be used to genetically manipulate gene expression in tumor cells, tumor stromal cells or both.

We have confirmed that Shh-induced rapid tumor growth also occurs in bi-clonal xenograft tumors created by co-injecting LNCaP cells with UGSM-2 cells.

We have shown that paracrine Hh signaling is the primary mechanism of Shh-induced tumor growth.

We have generated a set of retroviral expression constructs containing Shh pathway genes for use in determining the role of each of these genes in Shh accelerated tumor growth. We have generated UGSM and LNCaP cell lines stably expressing these genes.

We have generated stromal cell lines lacking Gli1 and Gli2 for the purpose of identifying stromal Gli1 and Gli2 roles in Shh-induced tumor growth.

We have identified Noggin as a gene overexpressed in stroma of LN-Shh tumors. Stromal Noggin overexpression alone is not sufficient to accelerate tumor growth.

REPORTABLE OUTCOMES:

A manuscript which describes the isolation and characterization of the UGSM-2 cell line is in press for publication in Prostate (manuscript attached).
A manuscript examining hedgehog signaling in human prostate cancer cell lines has been accepted for publication in the Journal of Urology (manuscript attached).
A review of hedgehog signaling in prostate cancer has been accepted for publication in the Journal of Urology (manuscript attached).

Invited Speaker – SBUR December, 2005 Miami Beach, Florida

SBUR Keynote Speaker Award, 2005 Miami Beach, Florida

CONCLUSIONS:

We are making good progress on the approved aims of the grant and I anticipate no significant changes in our research plan.

REFERENCES:

None

APPENDIX:

Manuscripts attached

Author Proof

Isolation and Characterization of an Immortalized Mouse Urogenital Sinus Mesenchyme Cell Line

Aubie Shaw,[1] John Papadopoulos,[2] Curtis Johnson,[2] and Wade Bushman[2]*

[1]*McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin*
[2]*Department of Surgery, University of Wisconsin, Madison, Wisconsin*

BACKGROUND. Stromal-epithelial signaling plays an important role in prostate development and cancer progression. Study of these interactions will be facilitated by the use of suitable prostate cell lines in appropriate model systems.

METHODS. We have isolated an immortalized prostate mesenchymal cell line from the mouse E16 urogenital sinus (UGS). We characterized its expression of stromal differentiation markers, response to androgen stimulation, ability to induce and participate in prostate morphogenesis, response to Shh stimulation, and interaction with prostate epithelial cells.

RESULTS. UGSM-2 cells express vimentin and smooth muscle actin, but not the mature smooth muscle markers myosin and desmin. This expression profile is consistent with a myofibroblast phenotype. Unlike other fibroblasts such as 3T3, UGSM-2 cells express androgen receptor mRNA and androgen stimulation increases proliferation. UGSM-2 cells are viable when grafted with embryonic UGS under the renal capsule and participate in glandular morphogenesis, but are not capable of inducing prostate morphogenesis of isolated UGS epithelium. Co-culture of UGSM-2 cells with human BPH-1 cells or co-grafting in vivo results in organized clusters of BPH-1 cells surrounded by a mantle of UGSM-2 cells. UGSM-2 cells are responsive to Sonic hedgehog (Shh), an important signaling factor in prostate development, and mimic the transcriptional response of the intact UGS mesenchyme. In co-cultures with BPH-1, UGSM-2 cells exhibit a robust transcriptional response to Shh secreted by BPH-1.

CONCLUSIONS. UGSM-2 is a urogenital sinus mesenchyme cell line that can be used to study stromal-epithelial interactions that are important in prostate biology. *Prostate 9999: 1–12, 2005.* © 2005 Wiley-Liss, Inc.

KEY WORDS: **stromal-epithelial interactions; androgen; sonic Hedgehog; prostate development; mesenchyme**

INTRODUCTION

The prostate develops from a specific region of the endodermal urogenital sinus (UGS) termed the prostatic anlagen. Formation of the prostatic ducts begins at embryonic day 17 (E17) in the mouse when epithelial buds evaginate into the surrounding mesenchymal sheath. Discrete groups of buds define the origins of the anterior, dorsal and ventral lobes of the prostate. At the time of ductal budding, the UGS mesenchyme is composed of undifferentiated fibroblasts and myofibroblasts. As the buds elongate, they lumenalize to form true secretory ducts connected to the urethral lumen and branch to form a highly complex ductal tree. As the ducts grow, they are surrounded by a sheath of mesenchyme, which differentiates to a periductal stroma comprised of smooth muscle cells and fibroblasts [1].

The embryonic mesenchyme and its adult descendent stroma have emerged as key regulators of prostatic growth and differentiation. In the UGS, mesenchymal cells express androgen receptors and act under the influence of androgens to induce prostatic differentiation of the endodermal epithelium [2,3].

Tissue recombination experiments have shown that the mesenchyme is the primary determinant of epithelial growth and differentiation [4]. In the adult prostate, there is regional heterogeneity within the ducts: the distal tips are encased in a delicate fibroblastic sheath, while the more proximal segments are surrounded by thicker sheaths rich in smooth muscle [5]. Androgen receptor expression is localized to the dense smooth muscle sheath surrounding epithelial ducts, whereas fibroblasts rarely express androgen receptors [6]. Smooth muscle is required for maintenance of epithelial secretory function [7] and loss of smooth muscle in the adult prostate is associated with cancer lesions and de-differentiation of epithelium [8].

Primary stromal cells from human prostate tissue have been used to discover factors that regulate smooth muscle differentiation and proliferation of prostate stroma, and to identify stromal-derived factors that regulate epithelial functions. Several prostate stromal cell lines have been generated, including rat NbF-I, mouse PSMC1, rat PS-1, human WPMY-1, human DuK50, and human PS30 cells [9–14]. Two rat UGS mesenchymal cell lines have been generated: rUGM and U4F1 [15,16]. To our knowledge, none of these cell lines is able to induce or participate in prostate morphogenesis.

The signaling interactions that regulate prostate ductal budding and branching morphogenesis have received considerable attention as the paradigm for understanding normal prostate growth regulation. These studies have demonstrated that the UGS mesenchyme is the target of several key signals, including testosterone, estrogen, and sonic hedgehog [17–19]. UGS mesenchyme is also the origin of several key morphogens including BMP-4, FGF-10, TGFβ [20–23], and Shh target genes such as IGFBP-6 (Lipinski et al., submitted[Q1]), which may regulate both epithelial and mesenchymal proliferation and differentiation. The complexity of these interactions is daunting. For the Shh pathway alone, there are three different Gli genes expressed in the UGS mesencyhme and each of these plays a unique role in the transcriptional response to Hh signaling [18]. Similar complexities exist in the multiplicity of receptor subtypes for BMP, TGFβ, and FGF signaling. To elucidate the complex regulation and crosstalk between these pathways in mesenchymal cells, we have developed an immortalized UGS mesenchymal cell line and demonstrated that it phenocopies the UGS mesenchyme response to Shh stimulation.

Several unique characteristics distinguish the mesenchyme of the urogenital sinus mesenchyme. These include responsiveness to androgen, the ability to induce prostate differentiation of isolated urogenital sinus epithelium, and responsiveness to morphogens such as Sonic hedgehog. UGSM-2 cells were found to be androgen responsive and to mimic the canonical response of urogenital sinus mesenchyme to Sonic Hedgehog. UGSM-2 cells did not induce morphogenesis of isolated UGS epithelium sheets, but when grafted together with the E16 UGS they did proliferate and become incorporated into the periductal stroma during glandular morphogenesis.

MATERIALS AND METHODS

Animals and Cell Lines

Balb/c 3T3 fibroblasts were obtained from ATCC and cultured according to ATCC guidelines. BPH-1 cells were obtained from Simon Hayward (Vanderbilt University, Nashville, TN) and maintained in RPMI + 25 mM HEPES + 10% FBS. UGSM-2 cells were maintained in DMEM/F12 + ITS + 10% FBS + 10^{-8} M DHT. Wild-type CD-1 and CD-1 nude mice were obtained from Charles River (Wilmington, MA). INK4a$^{-/-}$, β-actin-tva transgenic mice were obtained from Bart Williams (Van Andel Research Inst., Grand Rapids, MI). All animals were housed according to institutional animal use and care guidelines.

Isolation of UGSM-2 Cells

Immortalized UGSM-2 cells were derived from the urogenital sinus of an E16 male INK4a$^{-/-}$ tva transgenic mouse embryo. INK4a$^{-/-}$, β-actin-tva transgenic mice were provided by Bart Williams (Van Andel Research Institute, Grand Rapids, MI). UGS epithelium was separated from mesenchyme following trypsin digestion as described previously [24]. Mesenchyme was further dissociated into single cells by digestion in 0.5% collagenase. Dissociated mesenchymal cells were grown in DMEM + 15% FBS + 1% pen/strep until they reached confluence in a 6-well plate. Thereafter cells were grown in DMEM/F12 + 10% FBS + 1% pen/strep + 1% ITS + 10^{-8} M DHT (INK4 culture medium). The UGSM-2 clone was isolated from the mixed UGSM population by dilution cloning followed by ring cloning.

Growth Curve Analysis

UGSM-2 and 3T3 cells were plated at a density of 4×10^4 cells per well in 6-well plates in normal culture media containing 10% charcoal-stripped, dextran-treated fetal bovine serum, csFBS (Hyclone, Logan, UT). After 48 hr, cells were treated with 10^{-8} M R1881 or 0.1% ethanol in normal culture media containing 10% csFBS. Each day, cells were trypsinized, diluted 1:100 in Isoton II solution (Beckman, Fullerton, CA) and counted in triplicate using a ViCell XR viable cell counter (Beckman Coulter). No significant difference in

cell viabilities between treatments was noted. Doubling time was calculated by determining the time required to double the number of cells in linear mid-log phase.

Ploidy Analysis

UGSM-2 cells were determined to be tetraploid by comparison to ploidy number of known diploid cells: freshly isolated splenocytes from the spleen of a CD-1 mouse (Charles River, Wilmington, MA). Splenocytes and UGSM-2 cells were combined in the following three ways: (1) 2×10^6 splenocytes, (2) 0.5×10^6 splenocytes $+ 2 \times 10^6$ UGSM-2 cells, and (3) 2×10^6 UGSM-2 cells. Cells were pelleted and fixed in ice cold 70% ethanol for 30 min. Cells were then pelleted and resuspended in $33 \mu g/ml$ propidium iodide $+ 1 mg/ml$ RNase A $+ 0.2\%$ Nonidet P-40 in PBS. DNA content of cells was determined using a FACScan cytometer and analyzed using ModFitLT V3.0 software.

Immunocytochemistry

UGSM-2 cells were grown on Lab-Tek II chamber slides (Fisher, Pittsburgh, PA) and immunostained for vimentin, smooth muscle actin (SMA) or pan-cytokeratin (pan-CK). Slides were fixed in 4% paraformaldehyde and blocked in 5% normal goat serum in PBS. Anti-vimentin clone LN-6 (Sigma, St. Louis, MO), anti-smooth muscle actin monoclonal antibody clone 1A4 (Sigma) or anti-pan-cytokeratin monoclonal antibody (Zymed, South San Francisco, CA) was applied at a dilution of 1:200. Staining was visualized by incubating with goat anti-mouse Alexa 546 conjugated antibody (Molecular Probes, Eugene, OR) at a dilution of 1:200. Slides were mounted with Vectashield Hardset + DAPI mounting media (Vector, Burlingame, CA) and imaged using an Olympus model BX51 fluorescent microscope and Spot Advanced software v. 3.5.2.

RT-PCR

RNA was isolated from confluent cells using RNeasy mini kit (Qiagen, Valencia, CA) with optional on-column DNase digestion to eliminate contaminating DNA. Total RNA (1 µg) was reverse transcribed to generate cDNA using M-MLV reverse transcriptase (Invitrogen). Relative mRNA quantity was determined by real-time RT-PCR using iCycler instrumentation and software (BioRad, Hercules, CA). Primer sequences are listed in Table I. Primer sets whose name starts with 'm' are mouse-specific, while primer sets whose name starts with 'h' are human-specific. All sequences are listed in 5′–3′ orientation.

Co-Cultures

UGSM-2 and BPH-1 cells were plated at equal densities (1×10^6 cells each) in 25 cm^2 flasks coated with neutralized rat tail collagen [25]. Morphology of cells was observed and photographed over a 1-week period using a Nikon Eclipse TS100 inverted light microscope with a Spot Insight QE digital camera. RNA was prepared from 48 hr co-cultures as described above. Expression of Shh signaling targets Gli1 and Ptc1 was examined by RT-PCR.

Renal Capsule Grafts

For UGE + UGSM-2 grafts, E16 UGSs were separated into epithelium (UGE) and mesenchyme (UGM) using the method described previously [24]. UGE + UGSM-2 were combined and allowed to adhere together overnight on 0.6% agar plates containing INK4

TABLE I. Sequences of RT-PCR Primers

Target gene	Forward primer	Reverse primer
mGAPDH	AGCCTCGTCCCGTAGACAAAAT	CCGTGAGTGGAGTCATACTGGA
mSMA	ATCATGCGTCTGGACTTGG	AATAGCCACGCTCAGTCAGG
mVim	CCCCCTTCCTCACTTCTTTC	AAGAGTGGCAGAGGACTGGA
mDesmin	GTGAAGATGGCCTTGGATGT	TTGAGAGCAGAGAAGGTCTGG
mHCM	GCAGCTTCTACAGGCAAACC	CAAAGCGAGAGGAGTTGTCG
mAR	GTGAAGCAGGTAGCTCTGGG	GAGCCAGCGGAAAGTTGTAG
mCD31	CTGAGCCTAGTGTGGAAGCC	TACATCCATGTTCTGGGGGT
mGli1	GGAAGTCCTATTCACGCCTTGA	CAACCTTCTTGCTCACACATGTAAG
mPtc1	CTCTGGAGCAGATTTCCAAGG	TGCCGCAGTTCTTTTGAATG
mIGFBP6	AGCTCCAGACTGAGGTCTTCC	GAACGACACTGCTGCTTGC
mHIP	CCTGTCGAGGCTACTTTTCG	TCCATTGTGAGTCTGGGTCA
hGAPDH	CCACATCGCTCAGACACCAT	GCAACAATATCCACTTTACCAGAGTTAA
hGli1	AATGCTGCCATGGATGCTAGA	GAGTATCAGTAGGTGGGAAGTCCATAT
hPtc1	CGCTGGGACTGCTCCAAGT	GAGTTGTTGCAGCGTTAAAGGAA

culture medium. For UGS+UGSM-2 grafts, UGSs were dissected from E16 male CD-1 mouse embryos and chopped into five to six pieces, combined with UGSM-2 cells, and incubated overnight on agar plates prepared with INK4 culture media. For BPH-1 + UGSM-2 grafts, 500,000 UGSM-2 and 100,000 BPH-1 cells were resuspended in cold Matrigel (BD Biosciences, Becton, MA) and allowed to gel in sterile culture dishes. After 30 min, Matrigel beads containing cells were covered with INK4 culture medium and placed in a CO_2 incubator overnight. Recombinants were placed under the renal capsule of CD-1 adult male nude mice using the method outlined by Cunha, et al (http://mammary.nih.gov/tools/mousework/Cunha001/Pages/Navigation.html). After 1–4 weeks, grafts were harvested, fixed, and paraffin-embedded sections were prepared.

BrdU Pulse and Immunolabeling

BrdU labeling was used to trace UGSM-2 cells in renal grafts. Subconfluent UGSM-2 cells were incubated with 10 μM bromodeoxyuridine (BrdU) in normal culture media overnight. Overnight incubation with BrdU resulted in approximately 50% of cells with BrdU incorporated. Immunolabeling of cells in formalin fixed paraffin-embedded sections was accomplished using the BrdU Labeling and Detection Kit II (Roche, Indianapolis, IN). We used goat anti-mouse-Alexa 546 conjugated antibody (Molecular Probes) to visualize BrdU stained cells. Sections were co-stained for pan-cytokeratin (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:50 dilution. Pan-CK was visualized by incubating with goat anti-rabbit Alexa 488 conjugated antibody (Molecular Probes, Eugene, OR) at a dilution of 1:200. Sections were mounted with Vectashield Hardset mounting media + DAPI counterstain (Vector).

ShhTreatment

UGSM-2 cells were plated in 6-well plates at a density of 4×10^5 cells/well in complete media and allowed to attach overnight. The next day, cells were treated with 1 nm octylated N-Shh peptide (Curis, Inc., Cambridge, MA). After 48 hr, cells were lysed and RNA was collected. RNA was purified and prepared for RT-PCR as described above.

Shh Overexpression

A mammalian expression vector expressing human Shh driven by CMV promoter (pIRES2-hShh-EGFP) was constructed as described previously [26]. BPH-1 cells were transfected with pIRES2-hShh-EGFP vector or pIRES2-EGFP vector control (Clontech, Palo Alto, CA) using Lipofectamine 2000 (Invitrogen). BPH-1 cells stably overexpressing Shh/GFP were derived by fluorescence-activated cell sorting for GFP for 2 months after transfection. BPH-Shh cells stably express 50,000-fold more Shh mRNA than BPH-GFP or parent BPH-1 cells.

Statistical Analysis

An unpaired t-test was used to determine if significant differences exist between cell growth rates for untreated, testosterone or dihydrotestosterone treated cells. The Wilcoxon Rank Sum test was used to determine if there were significant differences in the gene expression responses to Shh treatment.

RESULTS

Isolation and Characterization of UGSM-2 Cells

Immortalized UGS mesenchymal (UGSM) cells were derived from a subline of the *INK4a* mouse, a transgenic knockout that lacks p16^{INK4a} and p19^{ARF}. Both p16^{INK4a} and p19^{ARF} are specific inhibitors of cyclin-dependent kinases Cdk4 and Cdk6 that regulate cell cycle progression [27]. Loss of p16^{INK4a} and p19^{ARF} allows mouse embryonic fibroblasts (MEFs) to escape cellular senescence. INK4a$^{-/-}$ MEFs spontaneously immortalize in culture [28]. UGSM cells were isolated by dissecting UGS mesenchyme from an E16 INK4a$^{-/-}$ mouse embryo (Fig. 1A). UGSM cells obtained in this fashion were propagated continuously without evidence of crisis. Immortalized mouse cells are typically tetraploid and these cells remained stably tetraploid for over 100 passages (data not shown). Several ring clones were derived and characterized. All exhibited a similar growth rate and morphology in culture and all responded to treatment with Sonic Hedgehog by upregulating transcription of the conserved Hh target genes Ptc and Gli1. One representative clonal cell line, UGSM-2, was selected for use in subsequent experiments. Like the parent mixed cell population, UGSM-2 cells were found to be stably tetraploid (Fig. 1B). Recent studies revealed that INK4a$^{-/-}$ MEFs can acquire chromosomal rearrangements at high passage [29]. To assess tumorigenicity, both the parent UGSM cell line and UGSM-2 cells were co-injected with Matrigel into the flanks of nude mice. No tumor formation was observed in any of 12 injections for each group of cells over 6 months observation, whereas co-injection of LNCaP cells with Matrigel at the same time yielded tumor formation at over 80% of sites injected within 6 weeks (data not shown). Sarcoma formation was observed when a mixed population of UGSM cells at high passage (>30) were injected into nude mice, however, we have never observed sarcoma formation with the UGSM-2 clone.
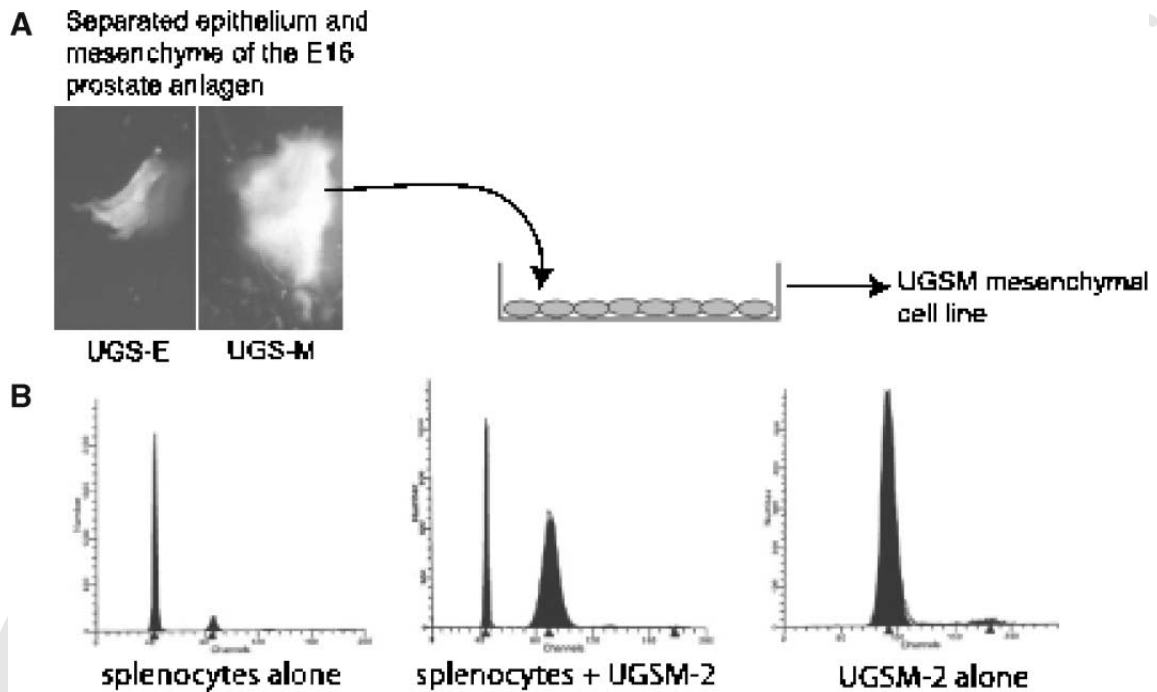
A Separated epithelium and mesenchyme of the E16 prostate anlagen

UGS-E   UGS-M

UGSM mesenchymal cell line

B

splenocytes alone   splenocytes + UGSM-2   UGSM-2 alone

Fig. I. Xxxxx[Q2].

UGSM-2 Cells Display a Myofibroblast Phenotype in Culture

The mesenchymal identity of UGSM-2 cells was established by characterizing expression of selected differentiation markers by RT-PCR and immunocyto-chemistry (Fig. 2). UGSM-2 cells express the stromal differentiation markers smooth muscle actin (SMA) and vimentin, and do not express either cytokeratins or the endothelial marker CD31/PECAM. The prostatic stroma contains cells that are classified as fibroblasts or smooth muscle, as well as cells termed myofibroblasts, which exhibit an intermediate phenotype. The profile of four stromal markers has been used to characterize cells as fibroblast (SMA−, vimentin+, desmin−, HCM−), myofibroblast (SMA+, vimentin+, desmin−, HCM−), or smooth muscle (SMA+, vimentin−, desmin+, HCM+) [30]. According to this classification UGSM-2 cells, which express SMA and vimentin, but do not express either desmin or heavy chain myosin (HCM) would be considered to exhibit a myofibroblast phenotype.

Growth Characteristics of the UGSM-2 Cell Line

Growth of many cell lines in culture is characterized by three phases: a lag phase while cells attach to the substrate; a log phase of exponential growth; and a plateau phase triggered by confluence and contact inhibition. UGSM-2 cell growth in culture exhibits all three phases of growth. The typical doubling time for UGSM-2 cells in normal culture media is 13 hr (Fig. 3A).

The presence of a plateau phase shows that UGSM-2 cells are contact inhibited and, indeed, the cells at confluence adopt a tight monolayer appearance (Fig. 5A).

Androgen Response of UGSM-2 Cells

The fetal urogenital sinus mesenchyme expresses androgen receptor and the androgen response of UGS mesenchyme is an important aspect of prostate biology. We examined androgen receptor expression by RT-PCR and found that UGSM-2 cells express the andro-gen receptor at levels comparable to the E16 UGS. Another fibroblast cell line that is not derived from the embryonic urogenital sinus, 3T3 fibroblasts, do not express androgen receptor (Fig. 2B). UGSM-2 cells are not dependent on androgen for survival or prolifera-tion (data not shown), however, their proliferation in culture is androgen sensitive. When we compared UGSM-2 growth in charcoal stripped serum supple-mented medium without exogenous steroid hormone or with 10^{-8} M synthetic androgen R1881, we found that UGSM-2 cells cultured in the presence of androgen grow at a significantly faster rate. 3T3 fibroblasts do not increase their proliferation rate in response to androgen (Fig. 3B). The same effects were seen with either 10^{-8} M testosterone or dihydrotestosterone (data not shown).

Participation of UGSM-2 in Prostate Morphogenesis

Our goal in developing the UGSM-2 cell line was to create a genetically modifiable cell line that could be used to study specific stromal-epithelial signaling
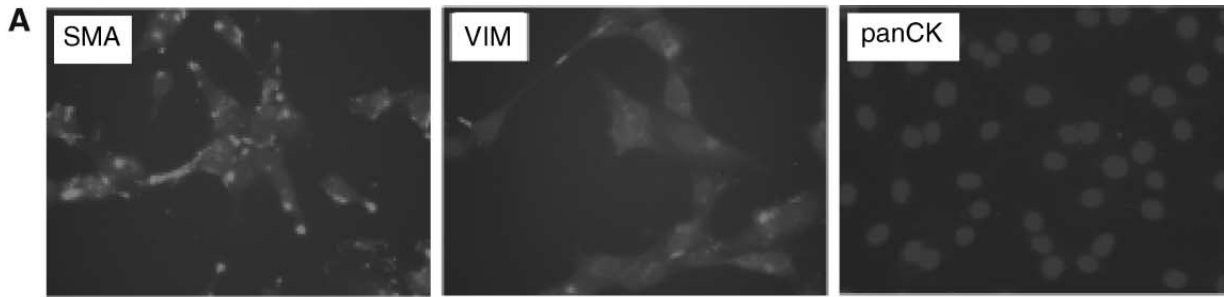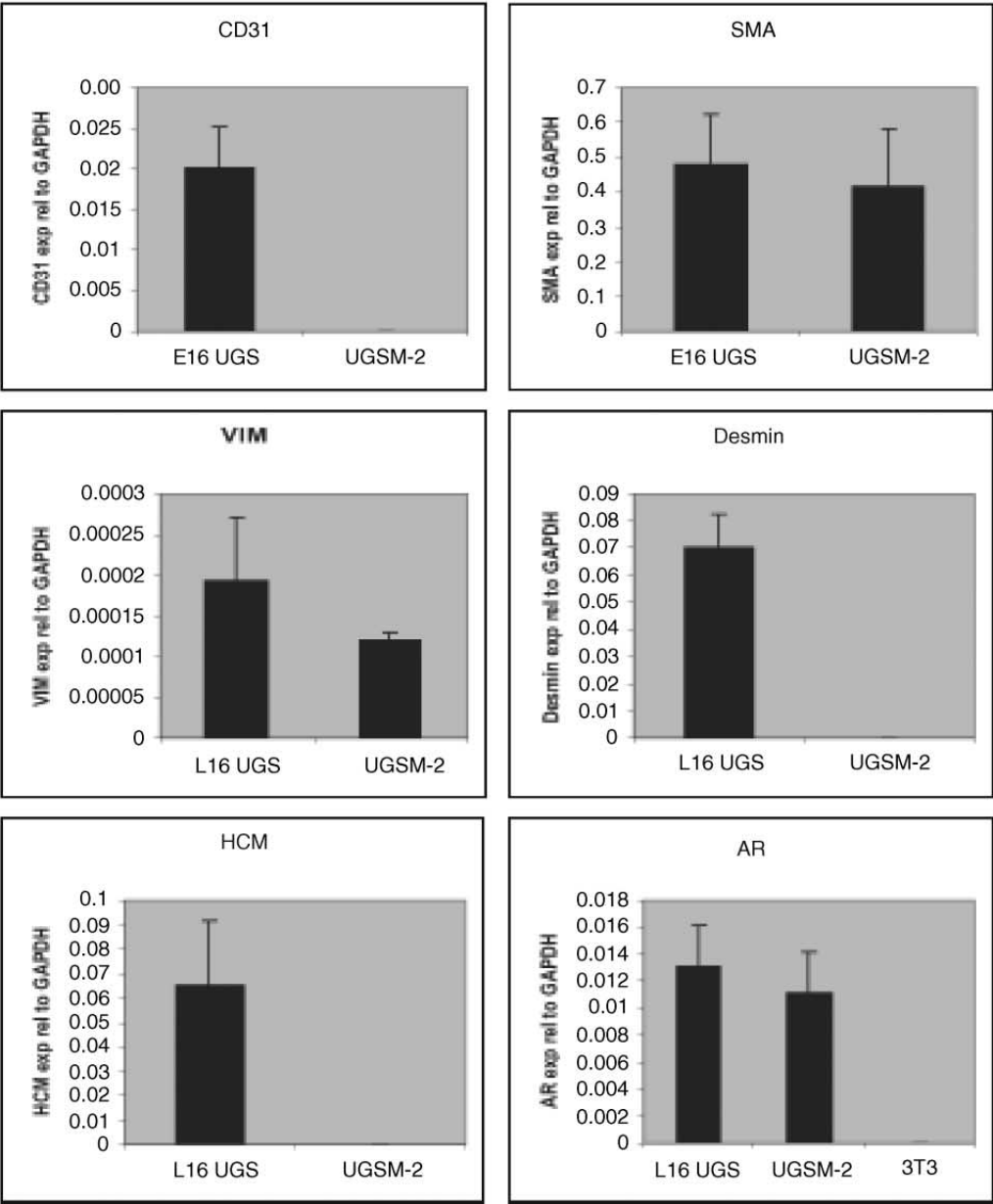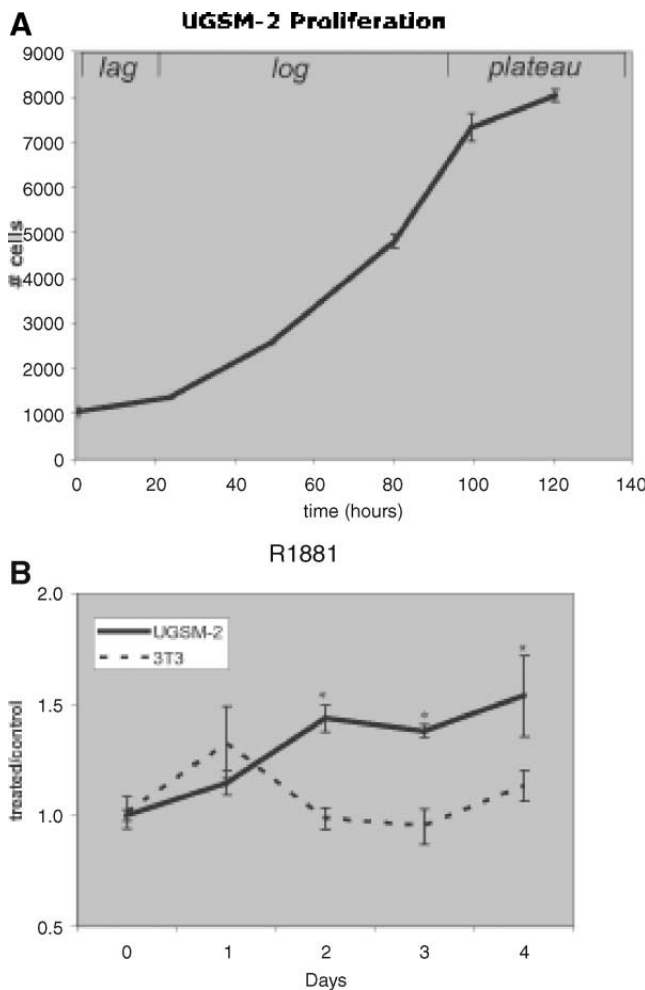
Fig. 2. Xxxxx.

Fig. 3. Xxxxx.

interactions in prostate development. We therefore examined the ability of UGSM-2 cells to mimic three attributes of E16 UGS mesenchyme (UGM): the capacity to induce prostatic differentiation in the UGS epithelium (UGE), the potential to form the stromal component of prostatic glands, and the ability to mimic the signaling interactions of urogenital sinus mesenchyme. To examine the ability of UGSM-2 cells to induce prostate morphogenesis we grafted UGSM-2 cells together with isolated E16 UGE sheets under the renal capsule of adult male nude mice. When retrieved 1 month later, the resulting grafts were much smaller than grafts composed of E16 UGE and E16 UGM (Fig. 4A) and histologic examination did not reveal any evidence of glandular morphogenesis (not shown). Therefore, UGSM-2 cells are unable to induce prostate development in this model system. To determine whether UGSM-2 cells can participate in glandular morphogenesis during prostate development, UGSM-2 cells were grafted with minced E16 UGS under the renal capsule of adult male nude mice. UGSM-2 cells

were pre-labeled with BrdU to trace their fate in matured UGS/prostate. The fate of UGSM-2 cells was examined after 1, 2, and 3 weeks of growth in vivo. UGS + UGSM-2 grafts had a similar size, gross morphology (data not shown) and histology (Fig. 4B) to minced E16 UGS implanted alone. Immunohistochemical staining for BrdU showed that BrdU-labeled UGSM-2 cells were present within the periductal stroma of the mature prostate tissue (Fig. 4C). The BrdU staining in nuclei of UGSM-2 cells exhibited varying degrees of speckling that increased from 1–3 weeks (data not shown). This was interpreted as indicating active UGSM-2 proliferation during growth of the grafted tissue.

To assess the interaction of UGSM-2 cells with adult prostate epithelial cells, UGSM-2 cells were co-cultured with human prostate epithelial BPH-1 cells. After 24 hr in co-culture, BPH-1 cells became organized into tight clusters surrounded by elongated UGSM-2 cells (Fig. 5A). When UGSM-2 cells were grafted together with BPH-1 cells under the renal capsule of adult male nude mice and the grafts examined one month later, the BPH-1 cells were organized into clusters surrounded by stromal cells very similar to those observed in co-culture (Fig. 5B). Mitotic figures were common in clusters, indicating active cell proliferation. Since BPH-1 cells injected alone do not form viable grafts, these observations suggest that UGSM-2 cells and BPH-1 can participate in a rudimentary process of cellular organization and that allows BPH-1 cells to survive and proliferate.

Shh Response of UGSM-2

To determine if the UGSM-2 cell line could accurately model the mesenchymal response to Shh signaling, we assayed gene expression in UGSM-2 cells treated with Shh peptide. When treated in cell culture with purified Shh peptide, UGSM-2 cells show robust activation of the conserved Hh target genes Gli1, Ptc1 and Hip. In addition, the Shh target gene IGFBP6, recently found to be upregulated in the UGS mesenchyme in response to Shh, was also induced (Fig. 6A). The three-fold increase in IGFBP6 expression after treatment with Shh is comparable to the response of the isolated E16 UGS mesenchyme to Shh [31]. To determine whether UGSM-2 cells would respond to Shh secreted by prostate epithelial cells in co-culture, we transfected BPH-1 cells with a Shh overexpression construct or GFP control vector (described in Fan et al., 2004). We cocultured UGSM-2 cells with the BPH-1 overexpressing or GFP control cells and analyzed Shh target gene expression using species-specific primers. This showed that overexpression of Shh by BPH-1 cells increased Gli1 and Ptc1 expression specifically in the
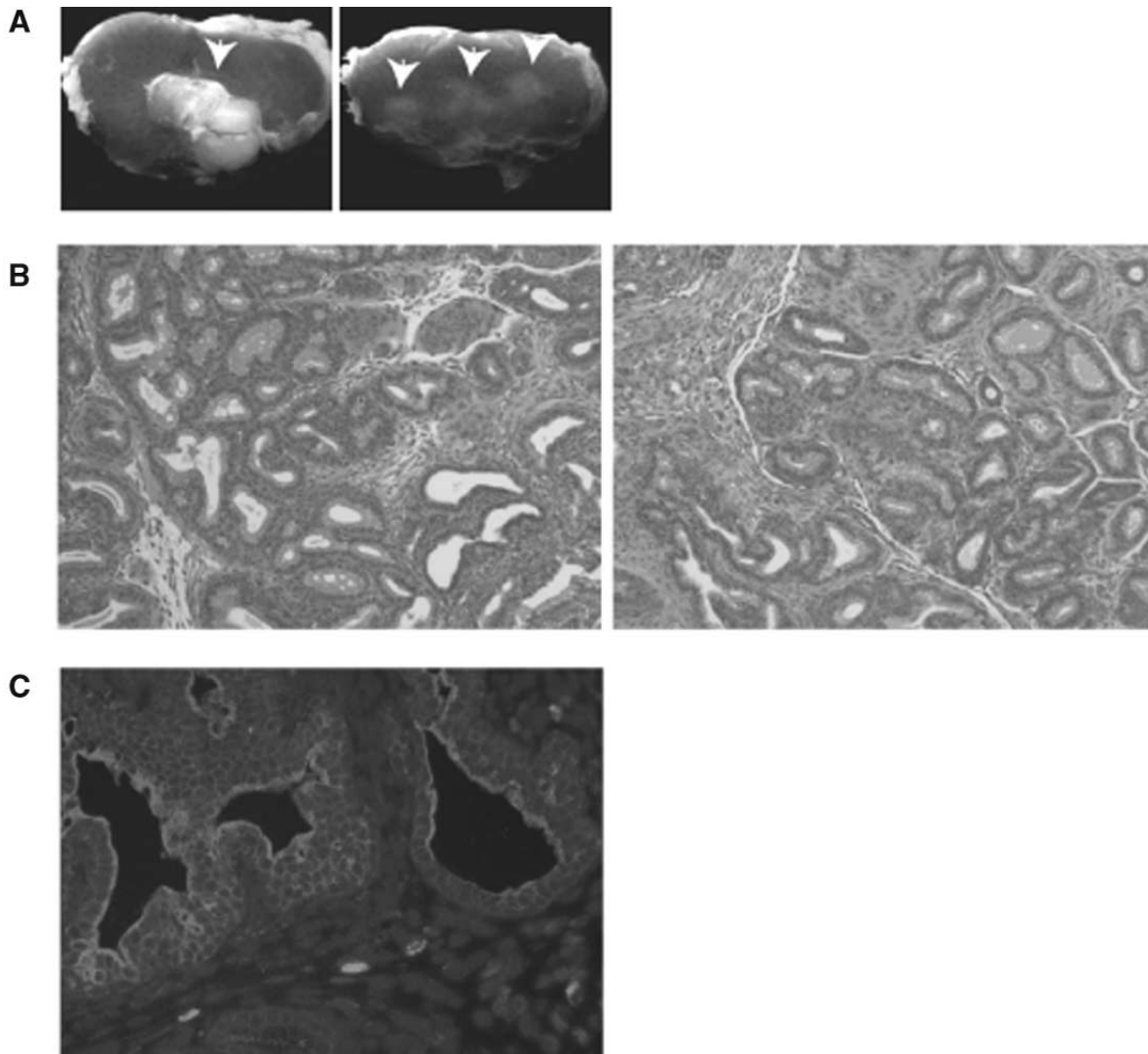
Fig. 4. Xxxxx.

UGSM-2 cells. There was no induction of Ptc and Gli1 in the BPH-1 cells (Fig. 6B). These experiments show that UGSM-2 cells in co-culture respond to a signaling ligand expressed by epithelial cells and can therefore mimic a stromal-epithelial interaction that plays an important role in prostate development.

CONCLUSIONS

Mechanistic studies of cell–cell interactions are facilitated by the use of genetically modified cell lines. Our long-term goal in developing the UGSM-2 cell line is to provide a tool for mechanistic studies of prostate development. We will use it to probe the mesenchymal signaling pathways that are important for prostate growth and differentiation. Urogenital sinus mesenchyme serves a critical role during prostate development as a medium for communication with developing epithelial glandular structures. Two of the signaling molecules involved in mesenchymal-epithelial communication during prostate development are androgen and Sonic hedgehog. The ability of UGSM-2 cells to respond to both of these molecules makes it an appropriate tool for mechanistic studies of androgen and Sonic hedgehog activities in prostate development.

We found that these cells could not induce prostate differentiation when co-transplanted with the isolated sheets of E16 UGS epithelium tissue. However, we cannot exclude the potential of these cells to exhibit inductive potential in other assays such as one that uses dissociated UGS epithelial cells grafted under the renal capsule [32]. When UGSM-2 cells were mixed with and co-transplanted with the whole UGS, they clearly did populate the mesenchyme/stroma of the subcapsular graft. In these grafts, UGSM-2 cells took up various positions within the stroma of mature prostate. Some
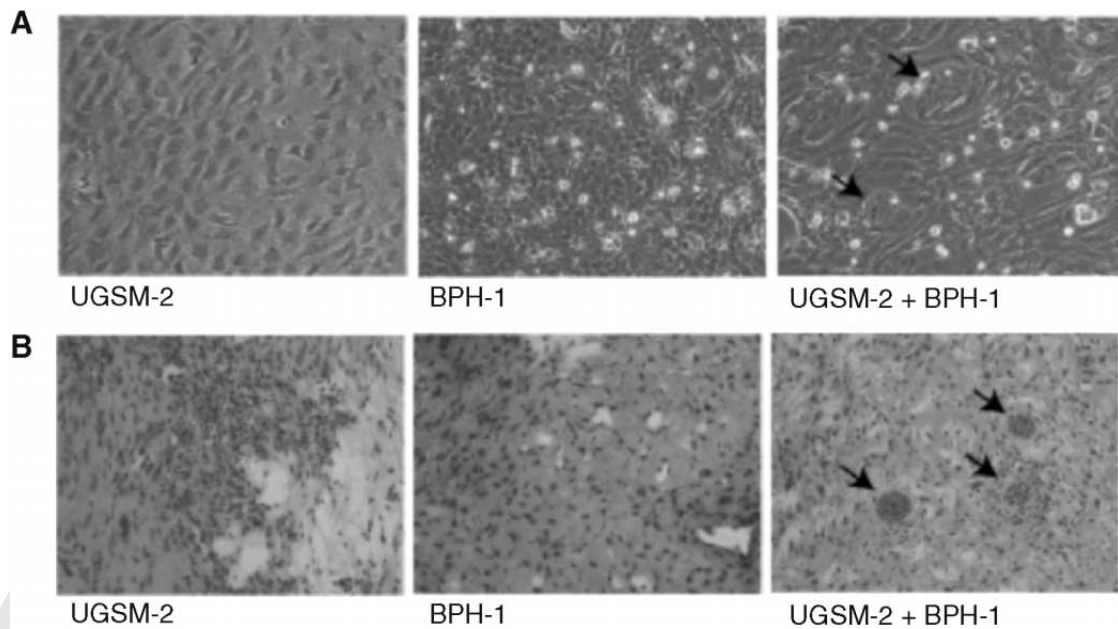
Fig. 5.   Xxxxx.

UGSM-2 cells were situated beside ductal epithelium, whereas others were embedded among other stromal cells in interductal stromal sheets. Although we have not analyzed stromal differentiation in these grafts, the ability of UGSM-2 cells to localize to different regions of the mature graft could indicate that they may take up both fibroblast and smooth muscle positions or functions in mature prostate tissue. Since UGSM-2 cells are able to occupy a stromal niche in developing UGS renal grafts, they may be used in in vivo gain and loss-of-function studies to examine the role of various gene products in early prostate development.

In addition to their ability to participate in prostate development, UGSM-2 cells form primitive acinar structures when either co-cultured or co-grafted with human BPH-1 prostate epithelial cells. Clustering of BPH-1 cells has been observed previously when co-cultured with primary fibroblasts derived from normal human prostate, but not with primary fibroblasts derived from human prostate tumors (Simon Hayward, personal communication). Cunha has shown that the inductive relationships between epithelium and mesenchyme are preserved between human and rodents [21]. Since the interactions between human epithelial cells and rodent mesenchymal cells are preserved, recombinants composed of human epithelium and UGSM-2 cells provide a useful model system for studying the role of these interactions in prostate development. An additional strength of this model is that we can distinguish signaling in mesenchyme and epithelium using species-specific RT-PCR. This dual species cell-based model therefore allows manipula-tion and analysis of gene expression in both epithelial and mesenchymal components to examine mesenchymal-epithelial interactions in vitro and in vivo.

In addition to their use in co-culture and xenograft models, UGSM-2 cells can be used as a cellular model to study mesenchymal signaling pathways that are important in prostate development. The first and most obvious use is to probe the molecular mechanisms of specific pathways. For example, we have used UGSM-2 cells to examine the concentration dependence and kinetics of Gli gene activation by Shh signaling (unpublished observations). The second is to use UGSM-2 cells in microarray studies to identify specific target genes of selected inductive signals. Finally, the immortalized UGSM cells can be used for genetic gain- and loss-of-function studies. Overexpression of selected genes in UGSM-2 cells may be engineered to examine the gain-of-function effect. It should be noted that the INK4a mutant was created by insertion of a neomycin resistance gene and cell lines derived from this mouse are neomycin resistant.

Therefore, an alternative method of selection must be used when these cells are transfected. We have successfully used adenovirus, retrovirus, and plasmid vectors with hygromycin or zeocin resistance selection to express genes of interest in UGSM-2. UGSM cells are particularly useful in studying genetic changes that are lethal, since harvest of UGSM cells at E16 allows for isolation of cells even from non-viable mutants. Indeed, we have developed UGSM cell lines from INK4a$^{-/-}$ mice bred to transgenic lines with mutations in various Shh signaling pathway components.
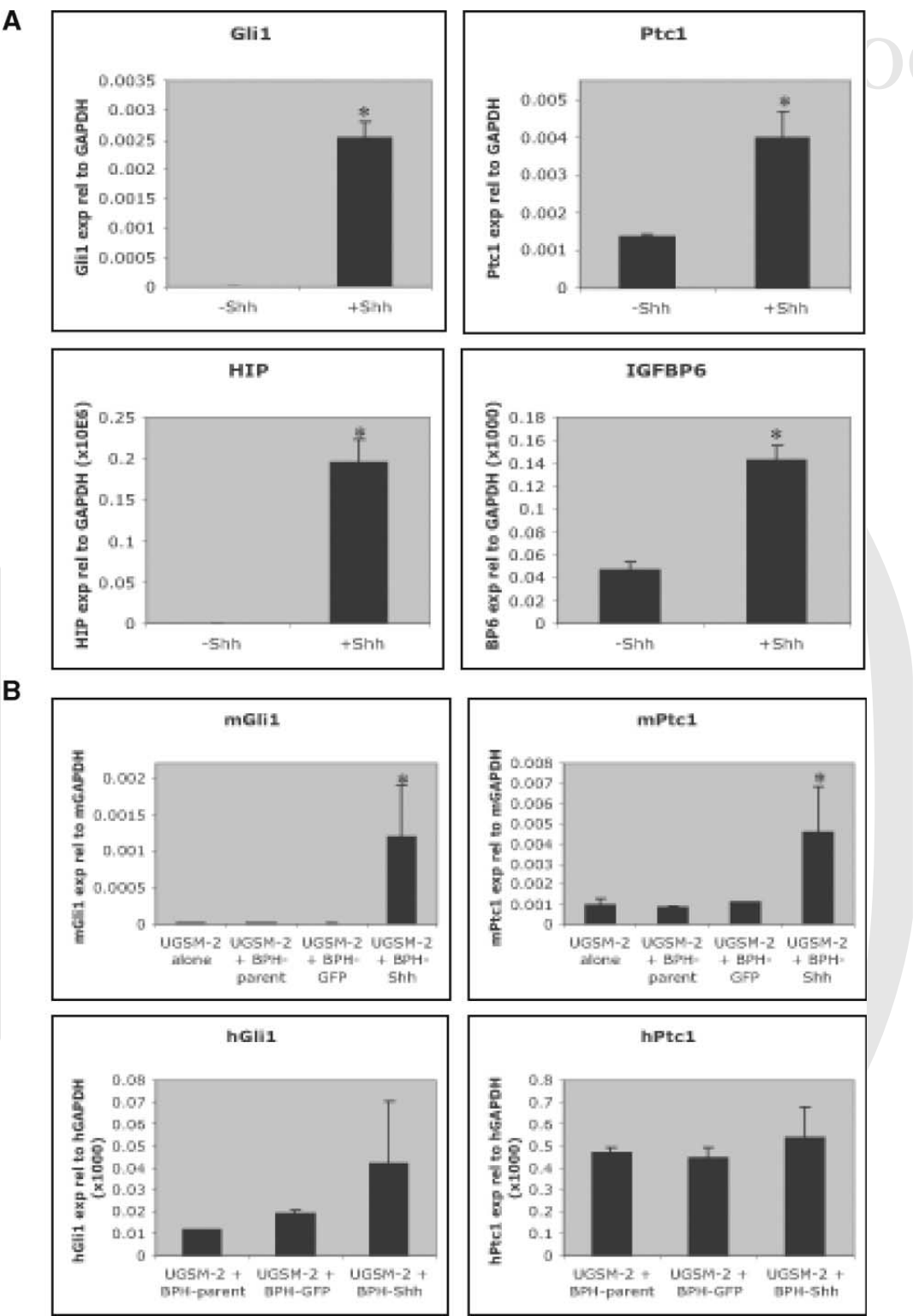
Fig. 6. Xxxxx.

The potential for immortalized stromal cell lines to become tumorigenic is well recognized. The INK4a$^{-/-}$ mutation produces impairment of G1 checkpoint control and the INK4a$^{-/-}$ mouse is prone to develop tumors in several mesenchymal tissues [28]. A recent report shows that INK4a$^{-/-}$ mouse embryonic fibroblasts display chromosomal rearrangements at high passage and develop the potential for sarcoma forma-

tion [29]. We have found that after 30 passages in culture, a mixed population of UGSM cells can form sarcomas when co-injected with Matrigel into nude mice. This can occur even while the cells remain contact inhibited and monolayer in culture (unpublished observations). However, we have never observed sarcoma formation with the UGSM-2 clonal cell line that was derived from the mixed UGSM population. Even so, we utilize the cells at low passage and perform sentinel grafts to monitor for sarcoma formation in all in vivo studies.

The UGSM-2 cell line and comparable cell lines derived from specific transgenic mutant mice will provide powerful tools to study signaling between prostate mesenchymal and epithelial cells. Using genetically modified UGSM cells in complementary cell-based assays, in vitro co-culture models and xenografts will allow detailed mechanistic studies of specific pathways and their influence on prostate development.

REFERENCES

1. Cunha GR, Donjacour AA, Cooke PS, Mee S, Bigsby RM, Higgins SJ, Sugimura Y. The endocrinology and developmental biology of the prostate. Endocr Rev 1987;8(3):338–362.

2. Boutin EL, Battle E, Cunha GR. The response of female urogenital tract epithelia to mesenchymal inductors is restricted by the germ layer origin of the epithelium: Prostatic inductions. Differentiation 1991;48(2):99–105.

3. Donjacour AA, Cunha GR. Assessment of prostatic protein secretion in tissue recombinants made of urogenital sinus mesenchyme and urothelium from normal or androgen-insensitive mice. Endocrinology 1993;132(6):2342–2350.

4. Cunha GR, Donjacour AA, Sugimura Y. Stromal-epithelial interactions and heterogeneity of proliferative activity within the prostate. Biochem Cell Biol 1986;64(6):608–614.

5. Donjacour AA, Cunha GR, Sugimura Y. Heterogeneity of structure and function in the mouse prostate. Prog Clin Biol Res 1987;239:583–600.

6. Hayward SW, Baskin LS, Haughney PC, Foster BA, Cunha AR, Dahiya R, Prins GS, Cunha GR. Stromal development in the ventral prostate, anterior prostate and seminal vesicle of the rat. Acta Anat (Basel) 1996;155(2):94–103.

7. Hayward SW, Haughney PC, Lopes ES, Danielpour D, Cunha GR. The rat prostatic epithelial cell line NRP-152 can differentiate in vivo in response to its stromal environment. Prostate 1999;39(3):205–212.

8. Wong YC, Tam NN. Dedifferentiation of stromal smooth muscle as a factor in prostate carcinogenesis. Differentiation 2002;70(9–10):633–645.

9. Chung LW, Chang SM, Bell C, Zhau HE, Ro JY, von Eschenbach AC. Co-inoculation of tumorigenic rat prostate mesenchymal cells with non-tumorigenic epithelial cells results in the development of carcinosarcoma in syngeneic and athymic animals. Int J Cancer 1989;43(6):1179–1187.

10. Gerdes MJ, Dang TD, Lu B, Larsen M, McBride L, Rowley DR. Androgen-regulated proliferation and gene transcription in a prostate smooth muscle cell line (PS-1). Endocrinology 1996; 137(3):864–872.

11. Price DT, Rudner X, Michelotti GA, Schwinn DA. Immortalization of a human prostate stromal cell line using a recombinant retroviral approach. J Urol 2000;164(6):2145–2150.

12. Roberson KM, Edwards DW, Chang GC, Robertson CN. Isolation and characterization of a novel human prostatic stromal cell culture: DuK50. In Vitro Cell Dev Biol Anim 1995;31(11):840–845.

13. Salm SN, Koikawa Y, Ogilvie V, Tsujimura A, Coetzee S, Moscatelli D, Moore E, Lepor H, Shapiro E, Sun TT, Wilson EL. Transforming growth factor-beta is an autocrine mitogen for a novel androgen-responsive murine prostatic smooth muscle cell line, PSMC1. J Cell Physiol 2000;185(3):416–424.

14. Webber MM, Trakul N, Thraves PS, Bello-DeOcampo D, Chu WW, Storto PD, Huard TK, Rhim JS, Williams DE. A human prostatic stromal myofibroblast cell line WPMY-1: A model for stromal-epithelial interactions in prostatic neoplasia. Carcinogenesis 1999;20(7):1185–1192.

15. Rowley DR. Characterization of a fetal urogenital sinus mesenchymal cell line U4F: Secretion of a negative growth regulatory activity. In Vitro Cell Dev Biol 1992;28A(1):29–38.

16. Zhau HE, Hong SJ, Chung LW. A fetal rat urogenital sinus mesenchymal cell line (rUGM): Accelerated growth and conferral of androgen-induced growth responsiveness upon a human bladder cancer epithelial cell line in vivo. Int J Cancer 1994;56(5):706–714.

17. Cunha GR, Chung LW. Stromal-epithelial interactions–I. Induction of prostatic phenotype in urothelium of testicular feminized (Tfm/y) mice. J Steroid Biochem 1981;14(12):1317–1324.

18. Lamm ML, Catbagan WS, Laciak RJ, Barnett DH, Hebner CM, Gaffield W, Walterhouse D, Iannaccone P, Bushman W. Sonic hedgehog activates mesenchymal Gli1 expression during prostate ductal bud formation. Dev Biol 2002;249(2):349–366.

19. Risbridger G, Wang H, Young P, Kurita T, Wang YZ, Lubahn D, Gustafsson JA, Cunha G. Evidence that epithelial and mesenchymal estrogen receptor-alpha mediates effects of estrogen on prostatic epithelium. Dev Biol 2001;229(2):432–442.

20. Donjacour AA, Thomson AA, Cunha GR. FGF-10 plays an essential role in the growth of the fetal prostate. Dev Biol 2003;261(1):39–54.

21. Hayward SW, Haughney PC, Rosen MA, Greulich KM, Weier HU, Dahiya R, Cunha GR. Interactions between adult human prostatic epithelium and rat urogenital sinus mesenchyme in a tissue recombination model. Differentiation 1998;63(3):131–140.

22. Lamm ML, Podlasek CA, Barnett DH, Lee J, Clemens JQ, Hebner CM, Bushman W. Mesenchymal factor bone morphogenetic protein 4 restricts ductal budding and branching morphogenesis in the developing prostate. Dev Biol 2001;232(2):301–314.

23. Raghow S, Shapiro E, Steiner MS. Immunohistochemical localization of transforming growth factor-alpha and transforming growth factor-beta during early human fetal prostate development. J Urol 1999;162(2):509–513.

24. Cunha G, Donjacour A. Mesenchymal-epithelial interactions: Technical considerations. In: Coffey D, Bruchovsky N, Gardner W, Resnick M, Karr J, editors. Current concepts and approaches to the study of prostate cancer. New York: AR Liss, Inc; 1987. pp 273–282.

25. Hallowes R, Bone E, Jones W. A new dimension in the culture of human breast. In: Richards R, Rajan K, editors. Tissue culture in medical research (II). Oxford: Pergamon Press; 1980. pp 213–220.

26. Fan L, Pepicelli CV, Dibble CC, Catbagan W, Zarycki JL, Laciak R, Gipp J, Shaw A, Lamm ML, Munoz A, Lipinski R, Thrasher JB, Bushman W. Hedgehog signaling promotes prostate xenograft tumor growth. Endocrinology 2004;145(8):3961–3970.

27. Sherr CJ, Roberts JM. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev 1995;9(10):1149–1163.

28. Serrano M, Lee H, Chin L, Cordon-Cardo C, Beach D, DePinho RA. Role of the INK4a locus in tumor suppression and cell mortality. Cell 1996;85(1):27–37.

29. Robertson S, Schoumans J, Looyenga B, Yuhas J, Zylstra C, Koeman J, Swiatek P, Teh B, Williams B. Spectral karyotyping of sarcomas and fibroblasts derived from Ink4a/Arf-deficient mice reveals chromosomal instability in vitro. Int J Oncol 2005; (in press[Q3]).

30. Tuxhorn JA, Ayala GE, Rowley DR. Reactive stroma in prostate cancer progression. J Urol 2001;166(6):2472–2483.

31. Lipinski RJ, Cook CH, Barnett DH, Gipp JJ, Peterson RE, Bushman W. Sonic hedgehog signaling regulates the expression of insulin-like growth factor binding protein-6 during fetal prostate development. Dev Dyn 2005;233(3):829–836.

32. Xin L, Ide H, Kim Y, Dubey P, Witte ON. In vivo regeneration of murine prostate from dissociated cell populations of postnatal epithelia and urogenital sinus mesenchyme. Proc Natl Acad Sci USA 2003;100(Suppl 1):11896–11903.

Q1: Please update the reference.

Q2: Please provide the figure legends.

Q3: Please update the reference.

WILEY

Publishers Since 1807

70 IRIS AVENUE, FLORAL PARK, NY 11001, USA

ELECTRONIC PROOF CHECKLIST, THE PROSTATE

IMMEDIATE RESPONSE REQUIRED
Please follow these instructions to avoid delay of publication.

☐ **READ PROOFS CAREFULLY**
- This will be your <u>only</u> chance to review these proofs.
- Please note that the volume and page numbers shown on the proofs are for position only.

☐ **ANSWER ALL QUERIES ON PROOFS** (Queries for you to answer are attached as the last page of your proof.)
- Mark all corrections directly on the proofs. Note that excessive author alterations may ultimately result in delay of publication and extra costs may be charged to you.

☐ **CHECK FIGURES AND TABLES CAREFULLY** (Color figures will be sent under separate cover.)
- Check size, numbering, and orientation of figures.
- All images in the PDF are downsampled (reduced to lower resolution and file size) to facilitate Internet delivery. These images will appear at higher resolution and sharpness in the printed article.
- Review figure legends to ensure that they are complete.
- Check all tables. Review layout, title, and footnotes.

☐ **COMPLETE REPRINT ORDER FORM**
- Fill out the attached reprint order form. It is important to return the form <u>even if you are not ordering reprints</u>. You may, if you wish, pay for the reprints with a credit card. Reprints will be mailed only after your article appears in print. This is the most opportune time to order reprints. If you wait until after your article comes off press, the reprints will be considerably more expensive.

RETURN ☐ **PROOFS**
☐ **REPRINT ORDER FORM**
☐ **CTA (If you have not already signed one)**

RETURN WITHIN 48 HOURS OF RECEIPT VIA FAX TO Mary Beth Puccio AT 516-437-3532

QUESTIONS? Mary Beth Puccio, Journal Production Editor
Phone: 201-748-8873
E-mail: mpuccio@wiley.com
Refer to journal acronym and article production number
(i.e., PROS 00-001 for *The Prostate* ms 00-001).

WILEY

Publishers Since 1807

COPYRIGHT TRANSFER AGREEMENT

Date:

To:

Production/Contribution
ID#_____
Publisher/Editorial office use only

Re: Manuscript entitled_____

_____ (the "Contribution")

for publication in _____ (the "Journal")

published by Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc. ("Wiley").

Dear Contributor(s):

Thank you for submitting your Contribution for publication. In order to expedite the publishing process and enable Wiley to disseminate your work to the fullest extent, we need to have this Copyright Transfer Agreement signed and returned to us as soon as possible. If the Contribution is not accepted for publication this Agreement shall be null and void.

A. COPYRIGHT

1. The Contributor assigns to Wiley, during the full term of copyright and any extensions or renewals of that term, all copyright in and to the Contribution, including but not limited to the right to publish, republish, transmit, sell, distribute and otherwise use the Contribution and the material contained therein in electronic and print editions of the Journal and in derivative works throughout the world, in all languages and in all media of expression now known or later developed, and to license or permit others to do so.

2. Reproduction, posting, transmission or other distribution or use of the Contribution or any material contained therein, in any medium as permitted hereunder, requires a citation to the Journal and an appropriate credit to Wiley as Publisher, suitable in form and content as follows: (Title of Article, Author, Journal Title and Volume/Issue Copyright © [year] Wiley-Liss, Inc. or copyright owner as specified in the Journal.)

B. RETAINED RIGHTS

Notwithstanding the above, the Contributor or, if applicable, the Contributor's Employer, retains all proprietary rights other than copyright, such as patent rights, in any process, procedure or article of manufacture described in the Contribution, and the right to make oral presentations of material from the Contribution.

C. OTHER RIGHTS OF CONTRIBUTOR

Wiley grants back to the Contributor the following:

1. The right to share with colleagues print or electronic "preprints" of the unpublished Contribution, in form and content as accepted by Wiley for publication in the Journal. Such preprints may be posted as electronic files on the Contributor's own website for personal or professional use, or on the Contributor's internal university or corporate networks/intranet, or secure external website at the Contributor's institution, but not for commercial sale or for any systematic external distribution by a third party (e.g., a listserve or database connected to a public access server). Prior to publication, the Contributor must include the following notice on the preprint: "This is a preprint of an article accepted for publication in [Journal title] © copyright (year) (copyright owner as specified in the Journal)". After publication of the Contribution by Wiley, the preprint notice should be amended to read as follows: "This is a preprint of an article published in [include the complete citation information for the final version of the Contribution as published in the print edition of the Journal]", and should provide an electronic link to the Journal's WWW site, located at the following Wiley URL: http://www.interscience.Wiley.com/. The Contributor agrees not to update the preprint or replace it with the published version of the Contribution.

2. The right, without charge, to photocopy or to transmit online or to download, print out and distribute to a colleague a copy of the published Contribution in whole or in part, for the Contributor's personal or professional use, for the advancement of scholarly or scientific research or study, or for corporate informational purposes in accordance with Paragraph D.2 below.

3. The right to republish, without charge, in print format, all or part of the material from the published Contribution in a book written or edited by the Contributor.

4. The right to use selected figures and tables, and selected text (up to 250 words, exclusive of the abstract) from the Contribution, for the Contributor's own teaching purposes, or for incorporation within another work by the Contributor that is made part of an edited work published (in print or electronic format) by a third party, or for presentation in electronic format on an internal computer network or external website of the Contributor or the Contributor's employer.

5. The right to include the Contribution in a compilation for classroom use (course packs) to be distributed to students at the Contributor's institution free of charge or to be stored in electronic format in datarooms for access by students at the Contributor's institution as part of their course work (sometimes called "electronic reserve rooms") and for in-house training programs at the Contributor's employer.

D. **CONTRIBUTIONS OWNED BY EMPLOYER**

1. If the Contribution was written by the Contributor in the course of the Contributor's employment (as a "work-made-for-hire" in the course of employment), the Contribution is owned by the company/employer which must sign this Agreement (in addition to the Contributor's signature), in the space provided below. In such case, the company/employer hereby assigns to Wiley, during the full term of copyright, all copyright in and to the Contribution for the full term of copyright throughout the world as specified in paragraph A above.

2. In addition to the rights specified as retained in paragraph B above and the rights granted back to the Contributor pursuant to paragraph C above, Wiley hereby grants back, without charge, to such company/employer, its subsidiaries and divisions, the right to make copies of and distribute the published Contribution internally in print format or electronically on the Company's internal network. Upon payment of the Publisher's reprint fee, the institution may distribute (but not resell) print copies of the published Contribution externally. Although copies so made shall not be available for individual re-sale, they may be included by the company/employer as part of an information package included with software or other products offered for sale or license. Posting of the published Contribution by the institution on a public access website may only be done with Wiley's written permission, and payment of any applicable fee(s).

E. **GOVERNMENT CONTRACTS**

In the case of a Contribution prepared under U.S. Government contract or grant, the U.S. Government may reproduce, without charge, all or portions of the Contribution and may authorize others to do so, for official U.S. Government purposes only, if the U.S. Government contract or grant so requires. (U.S. Government Employees: see note at end).

F. **COPYRIGHT NOTICE**

The Contributor and the company/employer agree that any and all copies of the Contribution or any part thereof distributed or posted by them in print or electronic format as permitted herein will include the notice of copyright as stipulated in the Journal and a full citation to the Journal as published by Wiley.

G. **CONTRIBUTOR'S REPRESENTATIONS**

The Contributor represents that the Contribution is the Contributor's original work. If the Contribution was prepared jointly, the Contributor agrees to inform the co-Contributors of the terms of this Agreement and to obtain their signature to this Agreement or their written permission to sign on their behalf. The Contribution is submitted only to this Journal and has not been published before, except for "preprints" as permitted above. (If excerpts from copyrighted works owned by third parties are included, the Contributor will obtain written permission from the copyright owners for all uses as set forth in Wiley's permissions form or in the Journal's Instructions for Contributors, and show credit to the sources in the Contribution.) The Contributor also warrants that the Contribution contains no libelous or unlawful statements, does not infringe on the rights or privacy of others, or contain material or instructions that might cause harm or injury.

CHECK ONE:

[_____]Contributor-owned work

_____   _____
Contributor's signature   Date

_____
Type or print name and title

_____   _____
Co-contributor's signature   Date

_____
Type or print name and title

ATTACH ADDITIONAL SIGNATURE PAGE AS NECESSARY

[_____]Company/Institution-owned work
    (made-for-hire in the
    course of employment)

_____   _____
Company or Institution (Employer-for-Hire)   Date

_____   _____
Authorized signature of Employer   Date

[_____]**U.S. Government work**

Note to U.S. Government Employees
A Contribution prepared by a U.S. federal government employee as part of the employee's official duties, or which is an official U.S. Government publication is called a "U.S. Government work," and is in the public domain in the United States. In such case, the employee may cross out Paragraph A.1 but must sign and return this Agreement. If the Contribution was not prepared as part of the employee's duties or is not an official U.S. Government publication, it is not a U.S. Government work.

[_____]**U.K. Government work (Crown Copyright)**

Note to U.K. Government Employees
The rights in a Contribution prepared by an employee of a U.K. government department, agency or other Crown body as part of his/her official duties, or which is an official government publication, belong to the Crown. In such case, the Publisher will forward the relevant form to the Employee for signature.
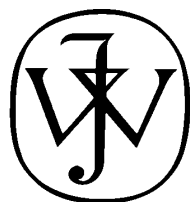
WILEY

Publishers Since 1807

THE PROSTATE

To: Mary Beth Puccio, Production Editor

Fax: 516-437-3532

From: Dr.

Date:

Re: *The Prostate,* ms #

Dear Ms. Mary Beth Puccio,

Attached please find corrections to ms# _____. Please contact me should you have any difficulty reading this fax at the numbers listed below.

Office phone:
Email:
Fax:
Lab phone:

Thank you,

Dr.

E-proofing feedback comments:

JW WILEY

Publishers Since 1807

REPRINT BILLING DEPARTMENT • 111 RIVER STREET, HOBOKEN, NJ 07030
PHONE: (201) 748-6670; FAX: (201) 748-6825
E-MAIL: reprints@wiley.com
PREPUBLICATION REPRINT ORDER FORM

Please complete this form even if you are not ordering reprints. This form **MUST** be returned with your corrected proofs and original manuscript. Your reprints will be shipped approximately 4 weeks after publication. Reprints ordered after printing will be substantially more expensive.

JOURNAL   *THE PROSTATE*   VOLUME _____   ISSUE _____
TITLE OF MANUSCRIPT _____
MS. NO. _____   NO. OF PAGES _____   AUTHOR(S) _____

No. of Pages	100 Reprints	200 Reprints	300 Reprints	400 Reprints	500 Reprints
	$	$	$	$	$
1-4	336	501	694	890	1052
5-8	469	703	987	1251	1477
9-12	594	923	1234	1565	1850
13-16	714	1156	1527	1901	2273
17-20	794	1340	1775	2212	2648
21-24	911	1529	2031	2536	3037
25-28	1004	1707	2267	2828	3388
29-32	1108	1894	2515	3135	3755
33-36	1219	2092	2773	3456	4143
37-40	1329	2290	3033	3776	4528

**REPRINTS ARE ONLY AVAILABLE IN LOTS OF 100. IF YOU WISH TO ORDER MORE THAN 500 REPRINTS, PLEASE CONTACT OUR REPRINTS DEPARTMENT AT (201) 748-6670 FOR A PRICE QUOTE.

☐   Please send me   _____   reprints of the above article at $ _____
_

Please add appropriate State and Local Tax   (Tax Exempt $ _____
                                                     No._____)
for United States orders only.

                                                     Please add 5% Postage and Handling $ _____

                              TOTAL AMOUNT OF ORDER $ _____
**International orders must be paid in currency and drawn on a U.S. bank*
Please check one: ☐   Check enclosed   ☐   Bill me   ☐   Credit Card
If credit card order, charge to: ☐   American Express   ☐   Visa   ☐   MasterCard

Credit Card No _____   Signature _____   Exp. Date _____

BILL TO: **SHIP TO:**   (Please, no P.O. Box numbers)
Name _____   Name _____

Institution _____   Institution _____

Address _____   Address _____

_____   _____

Purchase Order No. _____   Phone _____   Fax _____

                                                E-mail _____

Softproofing for advanced Adobe Acrobat Users - NOTES tool
NOTE: ACROBAT READER FROM THE INTERNET DOES NOT CONTAIN THE NOTES TOOL USED IN THIS PROCEDURE.

Acrobat annotation tools can be very useful for indicating changes to the PDF proof of your article. By using Acrobat annotation tools, a full digital pathway can be maintained for your page proofs.

The NOTES annotation tool can be used with either Adobe Acrobat 4.0, 5.0 or 6.0. Other annotation tools are also available in Acrobat 4.0, but this instruction sheet will concentrate on how to use the NOTES tool. Acrobat Reader, the free Internet download software from Adobe, DOES NOT contain the NOTES tool. In order to softproof using the NOTES tool you must have the full software suite Adobe Acrobat 4.0, 5.0 or 6.0 installed on your computer.

Steps for Softproofing using Adobe Acrobat NOTES tool:

1. Open the PDF page proof of your article using either Adobe Acrobat 4.0, 5.0 or 6.0. Proof your article on-screen or print a copy for markup of changes.

2. Go to File/Preferences/Annotations (in Acrobat 4.0) or Document/Add a Comment (in Acrobat 6.0 and enter your name into the "default user" or "author" field. Also, set the font size at 9 or 10 point.

3. When you have decided on the corrections to your article, select the NOTES tool from the Acrobat toolbox and click in the margin next to the text to be changed.

4. Enter your corrections into the NOTES text box window. Be sure to clearly indicate where the correction is to be placed and what text it will effect. If necessary to avoid confusion, you can use your TEXT SELECTION tool to copy the text to be corrected and paste it into the NOTES text box window. At this point, you can type the corrections directly into the NOTES text box window. **DO NOT correct the text by typing directly on the PDF page.**

5. Go through your entire article using the NOTES tool as described in Step 4.

6. When you have completed the corrections to your article, go to File/Export/Annotations (in Acrobat 4.0) or Document/Add a Comment (in Acrobat 6.0).

7. **When closing your article PDF be sure NOT to save changes to original file.**

8. To make changes to a NOTES file you have exported, simply re-open the original PDF proof file, go to File/Import/Notes and import the NOTES file you saved. Make changes and re-export NOTES file keeping the same file name.

9. When complete, attach your NOTES file to a reply e-mail message. Be sure to include your name, the date, and the title of the journal your article will be printed in.

AUTOCRINE HEDGEHOG SIGNALING IS NOT INVOLVED IN GROWTH OF HUMAN PROSTATE CANCER CELL LINES IN VITRO

JINGXIAN ZHANG, ROBERT LIPINSKI, AUBIE SHAW, JERRY GIPP AND WADE BUSHMAN

From the Department of Surgery and the McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin

Please send correspondence to: Department of Surgery, University of Wisconsin, 600 Highland Avenue, Madison, WI 53792 (telephone: 608-265-8705, FAX: 608-265-8133, e-mail: bushman@surgery.wisc.edu).

Running title: Hh signaling in prostate cancer cell lines

Key words: hedgehog pathway, prostate cancer, cell lines, cyclopamine

Abbreviations: Hedgehog; Hh, Patched; Ptc, Sonic Hedgehog; Shh, Indian Hedgehog; Ihh, Smoothened; Smo, Hedgehog-Interacting Protein ; Hip, Suppressor of Fused; SuFu, m; mouse gene, h; human gene

Word count: 2523

ABSTRACT

Introduction: Several reports have highlighted the role of Hh pathway activation in prostate cancer growth, but the relative contributions of autocrine and paracrine signaling remain unclear. Divergent reports of the presence or absence of autocrine signaling in established human prostate cancer cell lines has not clarified the matter.

Materials and Methods: We comprehensively characterized the expression of Hh pathway genes in three prostate cancer cell lines (LNCaP, PC3 and 22RV1) and examined their response to Shh ligand and to the Hh pathway inhibitor cyclopamine. Results: Expression of Hh ligand, Ptc and Gli1 in all three cell lines is lower than the level of expression in normal human prostate tissue. All three cell lines exhibited Hh target gene activation when transfected with an activated form of Gli2, but none showed a detectable transcriptional response to Hh ligand or to transfection with an activated form of Smo. Further, treatment with the Hh pathway inhibitor cyclopamine did not inhibit Hh target gene expression in any of the three prostate cancer cell lines even though cyclopamine did inhibit proliferation in culture.

Conclusions: LNCaP, PC3 and 22RV1 show no evidence of autocrine signaling by ligand dependent mechanisms and cyclopamine-mediated inhibition of growth in culture occurs independent of any discernable effect on canonical Hh pathway activity.

INTRODUCTION

Hedgehog (Hh) signaling is required for normal prostate development[1-7] and appears to exert both autocrine and paracrine signaling activities. The Hh ligands Shh and Ihh are expressed in the urogenital sinus epithelium and tips of the developing ducts. This is mirrored by expression of the Hh target genes Ptc and Gli1 in the adjacent mesenchyme and associated with concentrated epithelial expression of Ptc and Gli1 at the tips of the buds and growing ducts[6, 8].

Recent studies have shown that Hh signaling is active in human prostate cancer and accelerates tumor growth. However, the relative contribution of paracrine versus autocrine signaling to tumor growth and the role of ligand-dependent versus ligand-independent mechanisms of pathway activation in autocrine signaling remain unclear. Ligand-dependent pathway activation occurs when Hh ligand binds to its receptor Patched (Ptc), relieves inhibition of Smoothened (Smo) activity and activates Gli mediated target gene expression. Ligand-independent pathway activation results from mutations that abrogate regulatory mechanisms that prevent pathway activity in the absence of ligand. The studies reported here comprehensively examine ligand-dependent and ligand-independent pathway activity in the cell lines used most commonly to model Hh signaling in prostate cancer.

MATERIALS AND METHODS

Cell Lines. All the prostate cancer cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in the medium recommended by ATCC. BPH1 cells were a generous gift from Dr. Simon Hayward (Vanderbilt University, Nashville, TN) and were grown in RPMI 1640 medium plus 5% fetal calf serum (FCS). Four cDNA samples from independent human prostate epithelial cultures were kindly provided by Dr. David Jarrard (University of Wisconsin, Madison, WI). Human prostate total RNA and fetal brain total RNA were purchased from BD Biosciences (Palo Alto, CA). Human prostate total RNA was pooled from normal prostates of 32 Caucasian males ages 21-50. Human fetal brain total RNA is from normal fetal brains pooled from 21 spontaneously aborted male/female Caucasian fetuses,

ages 26-40 weeks. Cells were plated in a 24-well plate at a density of 1×10^5 cells/well. RNA was harvested after 3days for the comparison of Hh pathway gene expression in different cell lines. For the assay of gene expression after SHH/cyclopamine treatment, serum concentration was reduced to 1% after 1 day attachment, and either 1nM octylated N-SHH (Curis, Inc., Cambridge, MA) or 5uM cyclopamine (Toronto Research chemicals, Ontario, Canada) was added to the medium and RNA was harvested after 48 hours treatment. Each experiment was repeated three times. UGSM-2 cells[9] and MEFs were isolated in our laboratory.

Co-culture. UGSM-2 cells were plated at 1.6×10^5 cells/well in a 12 well plate. After 24 hours, cancer cells were set on top of UGSM-2 cells at the same density. 5uM cyclopamine or 1nM octylated N-SHH was added to the medium. RNA was harvested after 24 hours treatment.

Cell proliferation assay. Cells were set in a 24-well plate at a density of 20,000 cells/well and allowed to attach overnight. The concentration of FCS in the media was changed to 2%, and various concentrations of cyclopamine were added. Cells were grown for 4 days, harvested for RNA or trypsinized and counted by Vi-cell XR cell viability analyzer (Beckman Coulter, Fullerton, CA).

Adenovirus infection. Adenovirus constructs carrying Gli1-GFP, mGli2-GFP, ΔNmGli2-GFP, activated Smo*-GFP or GFP alone[10] were kindly provided by Dr. Chen-Ming Fan (Carnegie Inst, Baltimore, MD). Cells were plated in a 24-well plate at density of 1×10^5 cells/well. After 24 hours attachment, media was replaced with 1% FCS +/-adenovirus at a multiplicity of infection of 25-100 PFU/cells. Sonic hedgehog and cyclopamine were added at the same time. RNA was harvested and gene expression was determined as described below. Under these conditions, more than 90% of cells were infected according to GFP fluorescence by flow cytometry.

RNA isolation and real time RT-PCR. RNA was isolated using Qiagen (Valencia, CA) RNeasy RNA isolation Kits and subjected to on-column DNase digestion. cDNA was generated following standard protocols. Gene expression was assayed by Real Time RT-PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on BioRad iCycler (Hercules, CA) and using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard gene. Primer sequences used in this experiment are listed in Table I. Samples were run on 2% agarose gel.

Statistical analysis. An unpaired *t*-test was used to determine if there were statistically significant differences between treatment groups.

RESULTS

Hh pathway activity in prostate cancer cell lines. Comparison of Hh ligand expression in four prostate cancer cell lines (LNCaP, DU145, 22RV1, PC3) showed that ligand expression was highest in PC3 and lowest in LNCaP (Figure 1a). Shh and Ihh expression in PC3 was of the same order of magnitude as in the fetal brain but well below what is found in the normal adult prostate (Figure 1b). Four primary epithelial cell lines isolated from benign prostate tissue as well as BPH1 immortalized normal prostate epithelial cells exhibited expression intermediate between LNCaP and PC3 (Figure 1c).

Ptc and Gli1 are primary targets of Hh transcriptional activation. Ptc expression is highest in LNCaP and 22 RV1, intermediate in PC3 and lowest in DU145 cells (Figure 2a). Gli1 expression was similar in all cell lines (Figure 2a). Ptc and Gli1 expression in these cells lines was generally comparable to expression in the four primary epithelial cell lines and BPH-1,but well below the level of expression observed in the pooled normal prostate (Figure 2b).

Then we examined responsiveness of the tumor cell lines to exogenous Hh ligand. All cells were tested at 90% confluence and under a range of serum conditions. Treatment with 1nM Shh peptide elicited no detectable increase in the expression of either Ptc or Gli1 in any of the tumor cell lines tested (Figure 3).

Hh pathway responsiveness in prostate cancer cell lines. The tumor cell lines all express mRNA for the major components of the Hh signal transduction pathway (Figure 4a), however, the relative abundance of expression of each factor, determined by real-time RT-PCR, shows considerable variation (Figure 4b). It appears that the cells express all of the necessary machinery to respond to Shh, but do not respond. The lack of responsiveness to exogenous Shh ligand could result from 3 different mechanisms: a defect in the transmembrane receptor apparatus, a defect in the intracellular signal transduction mechanism or a specific block in the ability of Hh to induce Ptc and Gli1. To distinguish between these, we infected PC3 and 22RV1 cells with adenoviral vectors expressing either an activated form of hSmo (Smo*) or an activated form of mGli2 (ΔNmGli2). Smo* did not induce expression of Ptc and Gli1 in either cell line, whereas it induced robust Ptc and Gli1 expression in both mouse embryonic fibroblasts (MEFs) and UGSM-2 cells (Figure 5a insert and data not shown, respectively). In contrast, infection with an adenovirus vector expressing ΔNmGli2 induced robust expression of both Ptc and Gli1 in 22RV1. Infection of PC3 with the ΔNmGli2 induced only a small and statistically insignificant increase in Ptc expression but produced a robust induction of Gli1 expression (Figure 5b). This difference is likely due to Gli1 being a more sensitive marker of induction because of its lower basal expression in the absence of ligand.

Effect of cyclopamine on Hh signaling. The plant steroidal alkaloid cyclopamine inhibits Hh signaling by an interaction with Smo. We examined the effect of 5 uM cyclopamine on Ptc and Gli1 expression in these tumor cell lines. Regardless of whether the assay was performed in 10%, 1% or 0.1% FCS, we observed no significant reduction in Ptc or Gli1 expression in any tumor cell line (Figure 6). Control studies performed with UGSM-2 cells showed complete blockade of Hh signaling with 5 uM cyclopamine (Figure 6 <u>insert</u>). We also observed no effect of cyclopamine on the tumor cell lines when the assay was performed in the presence of exogenous Shh peptide (data not shown).

Effect of cyclopamine on tumor cell proliferation. We examined the effect of cyclopamine on growth of 22RV1, PC3 and LNCaP cells in culture. Treatment with 5 uM cyclopamine resulted in a decreased number of LNCaP cells after four days in culture, a slight decrease in the number of 22RV1 cells and no change in the number of PC3 cells (Figure 7). Treatment with 10uM cyclopamine significantly reduced the number of cells after four days in all three tumor cell lines, but this effect did not correlate with a significant inhibition of Hh pathway activity as measured by Ptc and Gli1 expression (Figure 7 insert).

It has been demonstrated that cyclopamine can inhibit growth of PC3 tumor xenografts. It has been assumed that this effect reflected the chemical inhibition of autocrine signaling in the xenograft, however, our studies do not demonstrate significant autocrine signaling in this cell line. To examine the possibility that cyclopamine might interfere with tumor growth by inhibiting Hh pathway activity in the tumor stroma, we examined the effect of cylopamine on PC3 tumor cells grown in co-culture with UGSM-2 cells. While cyclopamine did not affect expression of hPtc and hGli1 by the PC3 cells, it did efficiently inhibit mPtc and mGli1 expression by the UGSM-2 cells (Figure 8).

DISCUSSION

Our previous studies of Hh signaling in normal and neoplastic human prostate demonstrated comparable levels of expression of Hh ligand and Gli1 in specimens of benign and localized prostate cancer, with a suggestion of higher level expression in locally advanced and/or androgen independent prostate cancer. We demonstrated expression of Shh in the tumor epithelium with localization of Gli1 predominantly in the peri-glandular tumor stroma, and used the LNCaP xenograft to show that paracrine Shh signaling can accelerate tumor growth[11]. Recently, we have shown that the paracrine growth effect in response to Shh signaling depends upon the nature of the tumor stroma (unpublished observations). This may explain why Hh signaling may occur equally in the normal adult prostate and in prostate cancer but elicit dramatically different growth effects. Several other studies examining the expression of Shh in localized and metastatic prostate cancer suggested variably increased Shh expression in localized tumors exerting a combination of autocrine and paracrine signaling activity, and dramatically increased pathway activation in metastatic disease[11-14]. The possible contribution of autocrine signaling to tumor growth was examined by studying human tumor cell lines. LNCaP and PC3 cells were both found to express Shh, Ptc and Gli1[12, 13]. Cyclopamine inhibited proliferation of LNCaP cells in culture[13, 14] and was shown to inhibit Gli1 expression, suggesting that the effect was pathway specific[13]. Cyclopamine also inhibited proliferation of PC3 cells in culture[12-14]. Karhadkar et al[12] found that anti-Shh blocking antibody also inhibited PC3 proliferation in culture, but Sanchez et al[13] found that PC3 proliferation was unaffected by either anti-Shh blocking antibody or exogenous Shh. The discrepancy in these results has not been resolved and highlights uncertainty in the relative contributions of ligand dependent and ligand independent pathway activation to PC3 proliferation. The effect of chemical blockade of Hh signaling on tumor growth was examined by effect of cyclopamine administration on xenograft tumor growth[12]. Both PC3 and 22RV1 tumors showed a dose dependent inhibition of tumor growth and complete and sustained regression at the highest dose tested. The specificity of this effect was confirmed by showing that xenografts made with tumor cells over-expressing Gli1 were resistant to the anti-tumor effect of cyclopamine. These exciting studies suggested that autocrine pathway activity promotes tumor cell proliferation and that treatment with Hh inhibitors in vivo could produce sustained regression of established tumors. However, these studies produced discrepant findings regarding the relative contribution of ligand-dependent versus ligand-independent pathway activation in these cell lines and did not consider the possibility that cyclopamine's action could involve an effect on paracrine signaling.

Our studies show that LNCaP, DU145, PC3 and 22RV1 all exhibit Hh ligand expression and expression of Gli1 and Ptc. The level of ligand expression varies, with the highest level of mRNA expression present in PC3 being comparable to the robust level of expression observed in

the fetal brain. Even so, this is below the level of expression in a pooled normal prostate sample composed of 32 prostate specimens from men 21-50 years of age.   Similarly, the expression of Ptc and Gli1 in these cell lines is much lower than in the normal prostate.

Since the tumor cell lines express both Shh and Ihh, low level pathway activity could result from ligand-dependent autocrine pathway activation. However, our extensive studies of LNCaP, PC3 and 22RV1 found no evidence for a transcriptional response to Hh stimulation or cyclopamine inhibition. Our previous studies had shown that LNCaP was unresponsive to exogenous Shh[11], but the unresponsiveness of PC3 and 22RV1 was unexpected. To validate these observations, we examined the effect of intracellular pathway activation in PC3 and 22RV1 cells.   Infection with an adenoviral vector expressing activated Smo did not induce Ptc or Gli1 transcription in either cell line - arguing that the canonical Smo-mediated signal transduction pathway is non-functional. To ensure the validity of this negative result, we infected cells with an adenoviral vector expressing an activated form of Gli2 (ΔNmGli2) and demonstrated induction of Ptc and Gli in both cell lines.   In these cells, the Hh pathway appears to be nonfunctional at the level of Smo, but the transcriptional response to Gli2 remains intact. These studies, which demonstrate that the Hh signal transduction mechanism is defective in both PC3 and 22RV1, are clearly consistent with a lack of responsiveness to Hh ligand.

Cyclopamine has been shown to inhibit Hh signaling by interfering with pathway activation by Smo. We observed no changes in the expression of Hh target genes Ptc and Gli1 in LNCaP, PC3 or 22RV1 treated with 5uM cyclopamine under a range of culture conditions. These observations are consistent with the aforementioned idea that Smo-mediated Hh signaling is defective in these cells. Although these observations stand in contrast to the studies of Karhadkar et al[12], they examined the effect of cyclopamine on expression of a Gli-reporter construct, rather than expression of endogenous Ptc and Gli1. It is therefore possible that they observed an effect of cyclopamine that does not accurately reflect the effect of cyclopamine on the expression of endogenous target genes. Similarly, we observed that treatment of cells in culture with 10 uM cyclopamine decreased cell number but without any discernable effect on Hh pathway activity. These findings strongly suggest that inhibition of cell proliferation in vitro by cyclopamine is not the result of canonical Smo-mediated Hh pathway inhibition.

How can we reconcile these observations with previously published studies showing a dramatic effect of cyclopamine on PC3 and 22RV1 xenograft tumors? One explanation is that PC3 and 22RV1 cells growing in vivo exhibit a different phenotype and are susceptible to cyclopamine-mediated inhibition of canonical pathway activity. Another is that the effect of cyclopamine on xenograft tumor growth is mediated through an effect on stromal cells responding to Hh ligand produced by the tumor cells. This is supported by our co-culture studies and suggests that the effect of cyclopamine on paracrine signaling in xenograft tumors may be an important, but unrecognized effect in the previously published studies.

<div align="center">REFERENCES</div>

1.   Podlasek, C. A., Barnett, D. H., Clemens, J. Q. et al.: Prostate development requires Sonic hedgehog expressed by the urogenital sinus epithelium. Dev Biol, **209:** 28, 1999

2.   Lamm, M. L., Catbagan, W. S., Laciak, R. J. et al.: Sonic hedgehog activates mesenchymal Gli1 expression during prostate ductal bud formation. Dev Biol, **249:** 349, 2002

3.   Wang, B. E., Shou, J., Ross, S. et al.: Inhibition of epithelial ductal branching in the prostate by sonic hedgehog is indirectly mediated by stromal cells. J Biol Chem, **278:** 18506, 2003

4.   Freestone, S. H., Marker, P., Grace, O. C. et al.: Sonic hedgehog regulates prostatic growth and epithelial differentiation. Dev Biol, **264:** 352, 2003

5.   Berman, D. M., Desai, N., Wang, X. et al.: Roles for Hedgehog signaling in androgen production and prostate ductal morphogenesis. Dev Biol, **267:** 387, 2004

6.   Pu, Y., Huang, L., Prins, G. S.: Sonic hedgehog-patched Gli signaling in the developing rat prostate gland: lobe-specific suppression by neonatal estrogens reduces ductal growth and branching. Dev Biol, **273:** 257, 2004

7.   Doles, J., Shi, X., Bushman, W.: Functional Redundancy of Hedgehog Signaling Regulating Mouse Prostate Development. Dev. Biol, **in press**, 2005

8.   Gao, N., Ishii, K., Mirosevich, J. et al.: Forkhead box A1 regulates prostate ductal morphogenesis and promotes epithelial cell maturation. Development, **132:** 3431, 2005

9.   Shaw, A., Papadopoulos, J., Johnson, C. et al.: Isolation and Characterization of an Immortalized Mouse Urogenital Sinus Mesenchyme Cell Line. The prostate, **in press**, 2005

10.   Buttitta, L., Mo, R., Hui, C. C. et al.: Interplays of Gli2 and Gli3 and their requirement in mediating Shh-dependent sclerotome induction. Development, **130:** 6233, 2003

11.   Fan, L., Pepicelli, C. V., Dibble, C. C. et al.: Hedgehog signaling promotes prostate xenograft tumor growth. Endocrinology, **145:** 3961, 2004

12.   Karhadkar, S. S., Bova, G. S., Abdallah, N. et al.: Hedgehog signalling in prostate regeneration, neoplasia and metastasis. Nature, **431:** 707, 2004

13.   Sanchez, P., Hernandez, A. M., Stecca, B. et al.: Inhibition of prostate cancer proliferation by interference with SONIC HEDGEHOG-GLI1 signaling. Proc Natl Acad Sci U S A, **101:** 12561, 2004

14.   Sheng, T., Li, C., Zhang, X. et al.: Activation of the hedgehog pathway in advanced prostate cancer. Mol Cancer, **3:** 29, 2004
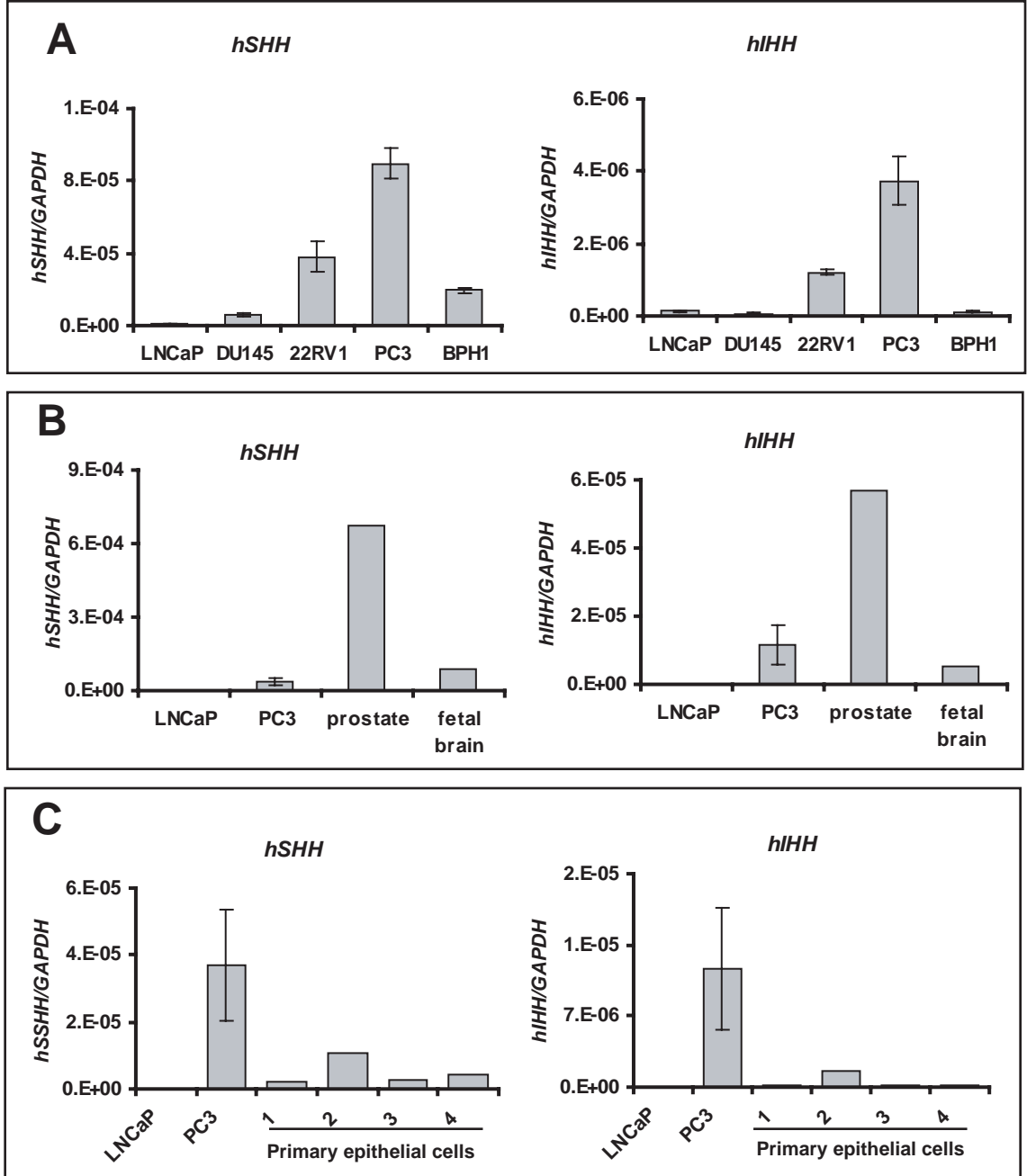
Figure 1 (A) Shh and Ihh expression in four prostate cancer cell lines (LNCaP, DU145, PC3 and 22RV1) and the immortalized BPH-1 cell line. (B) Comparison of expression in LNCaP and PC3 with expression in the human fetal brain and a pooled sample of normal adult prostate RNA. (C) Comparison of expression in LNCaP and PC3 with expression in four primary benign prostate epithelial cell lines.
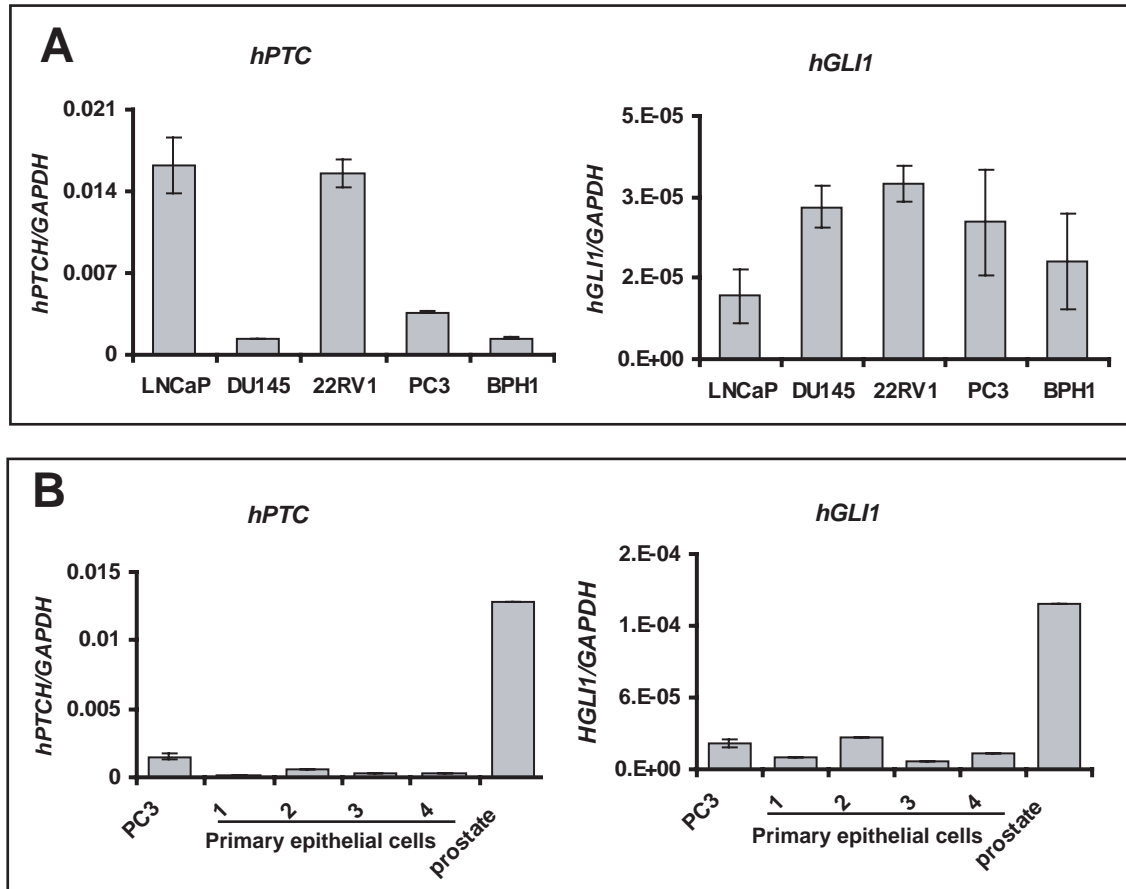
Figure 2 (A) Expression of the conserved Hh target genes Ptc and Gli1 in four prostate cancer cell lines (LNCaP, DU145, PC3 and 22RV1) and the immortalized BPH-1 cell line. (B) Comparison of Ptc and Gli1 expression in PC3 and four primary benign prostate epithelial cell lines.
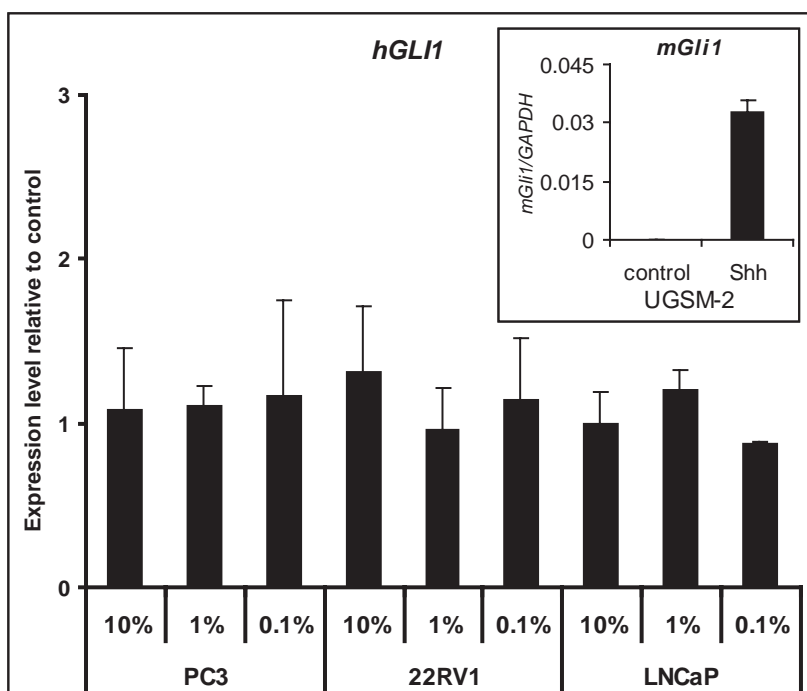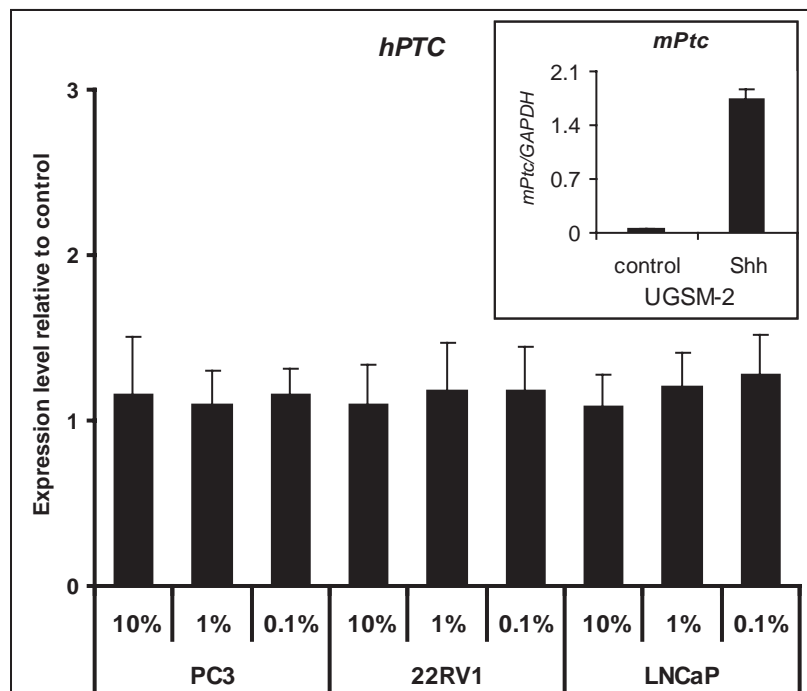
Figure 3 Lack of induction of Ptc1 and Gli1 expression in Shh treated PC3, 22RV1 and LNCaP cells. Shh treatment was in media supplemented with 10%, 1%, or 0.1% FCS for 48hr which is sufficient to significantly induce Ptc1 and Gli1 in UGSM-2 cells (0.1% FCS), $p<0.005$ (insert).
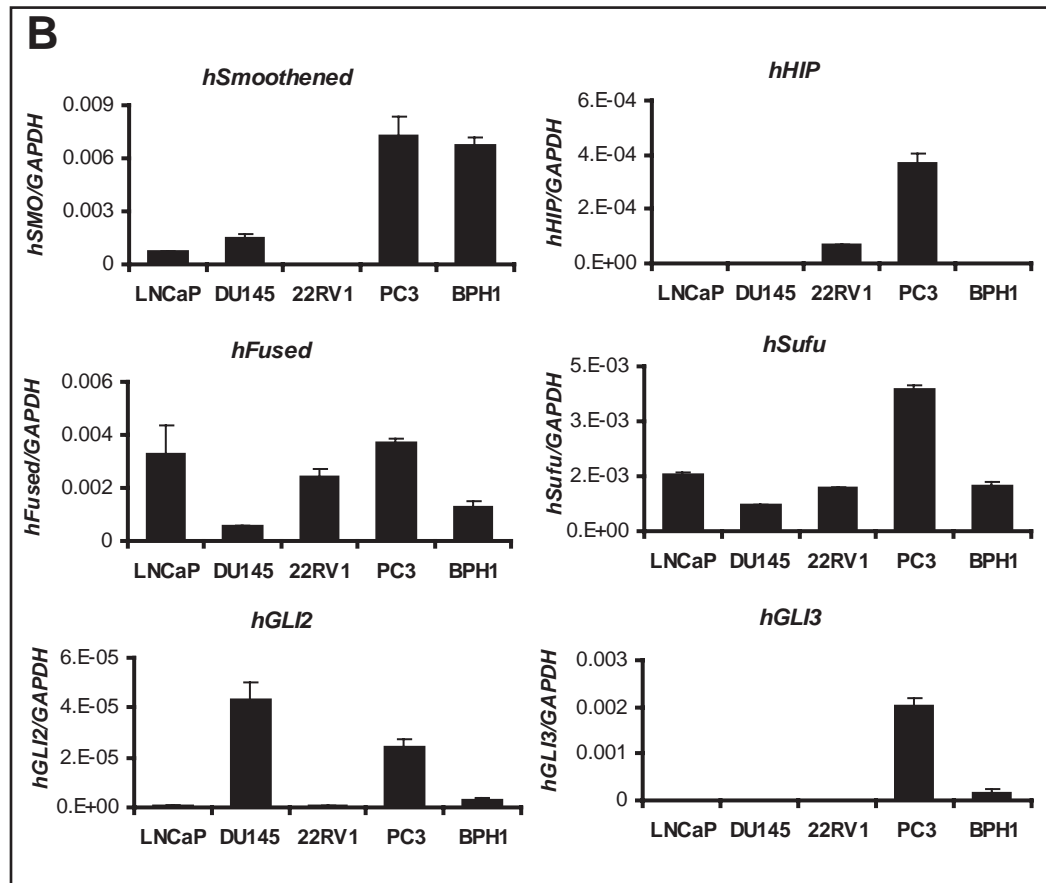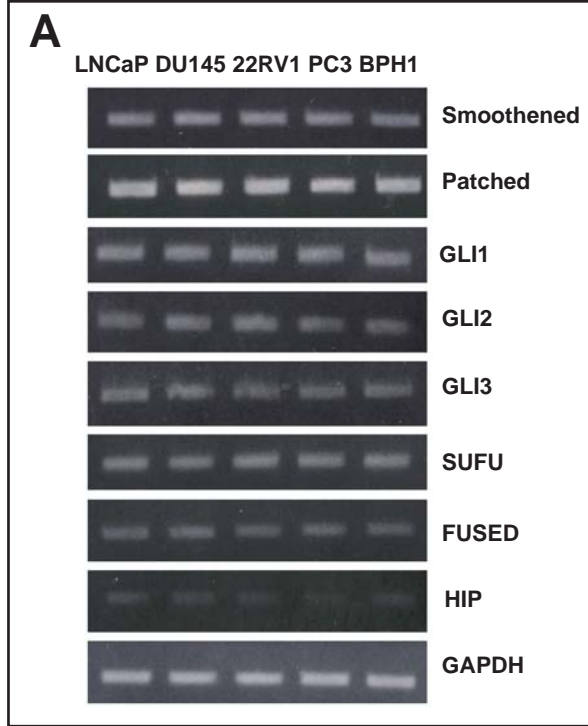
Figure 4 Expression of Hh pathway genes Smo, Ptc1, Gli1, Gli2, Gli3, SuFu, Fused and Hip in LNCaP, DU145, PC3, 22RV1 and BPH-1. (A) Resolution of RT-PCR products (40 cycles) on a 2% agarose gel using GAPDH as a loading control. (B) Quantitative real-time RT-PCR for the Hh pathway genes shows variations in the steady state levels of individual pathway components.
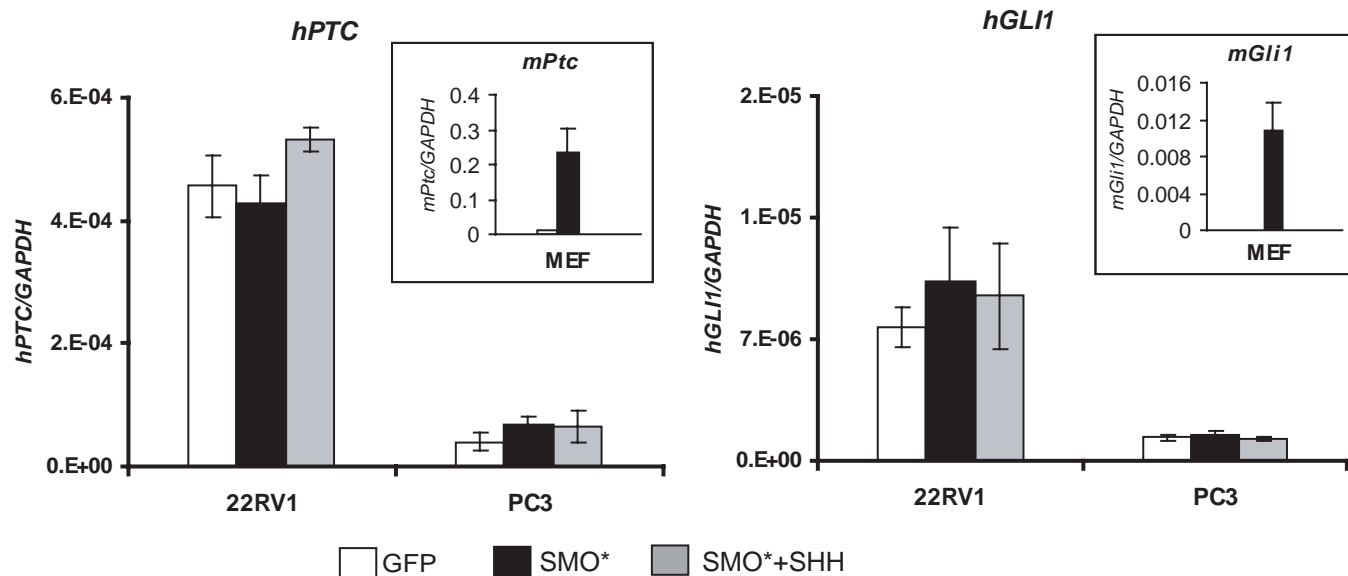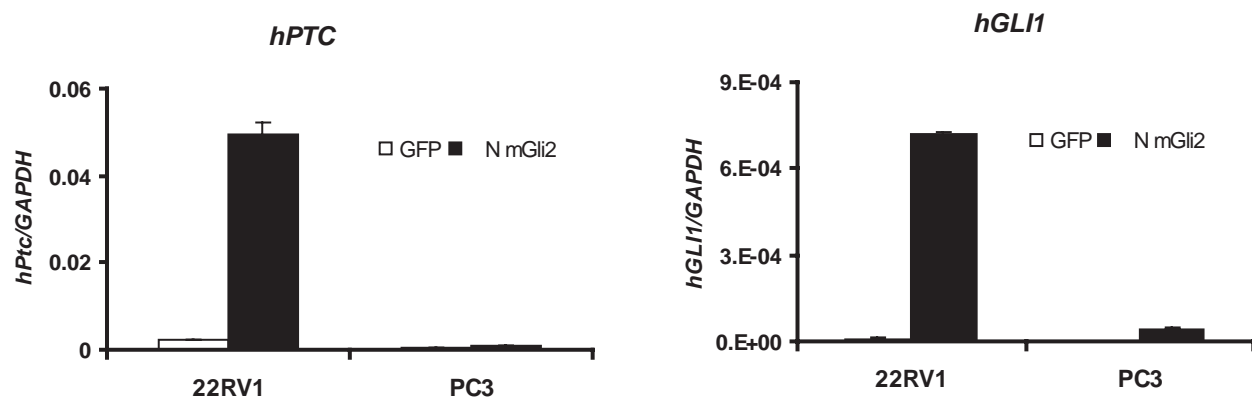
Figure 5 (A) Infection of PC3 and 22RV1 with a Smo* adenoviral vector did not activate expression of Hh target genes Ptc or Gli1, even when exogenous Shh was added. (insert A) Activation of Ptc1 and Gli1 is achieved in MEF cells under the same conditions (p<0.05). (B) Infection of PC3 and 22RV1 with a ?NmGli2 adenoviral vector induced Hh target gene expression. Both PC3 and 22RV1 exhibit significant increases in Gli1 expression (p<0.05); Ptc expression was significantly increased in 22RV1 cells (p<0.005) but not in PC3 cells (p=0.097). Adenovirus infection rates for all constructs was ~90%.
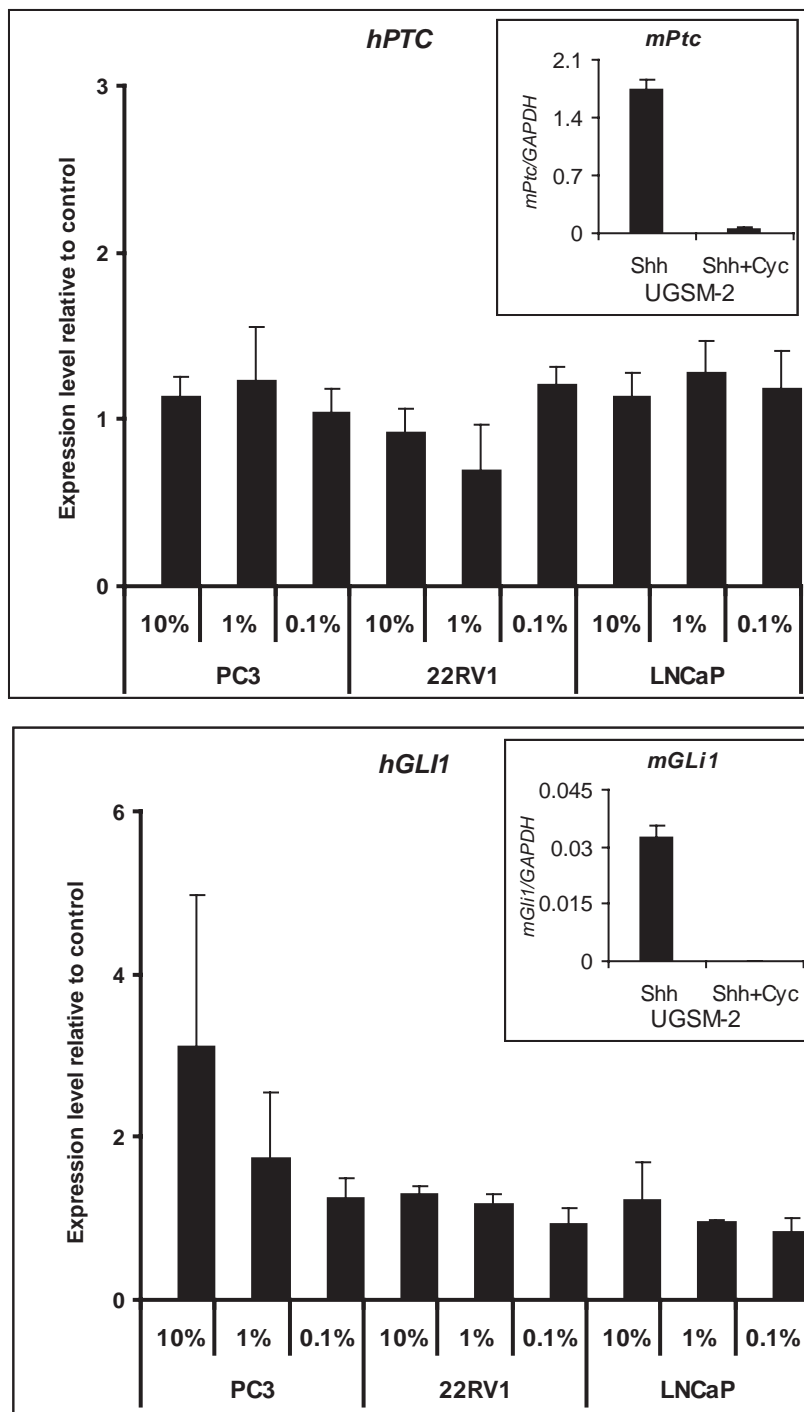
Figure 6 Cyclopamine treatment of PC3, 22RV1 and LNCaP cells in media supplemented with 10, 1, 0.1% FCS did not alter expression of the Hh target genes Ptc or Gli1. Target gene expression was induced by Shh and inhibited by 5uM cyclopamine in UGSM-2 cells p<0.005, (insert).
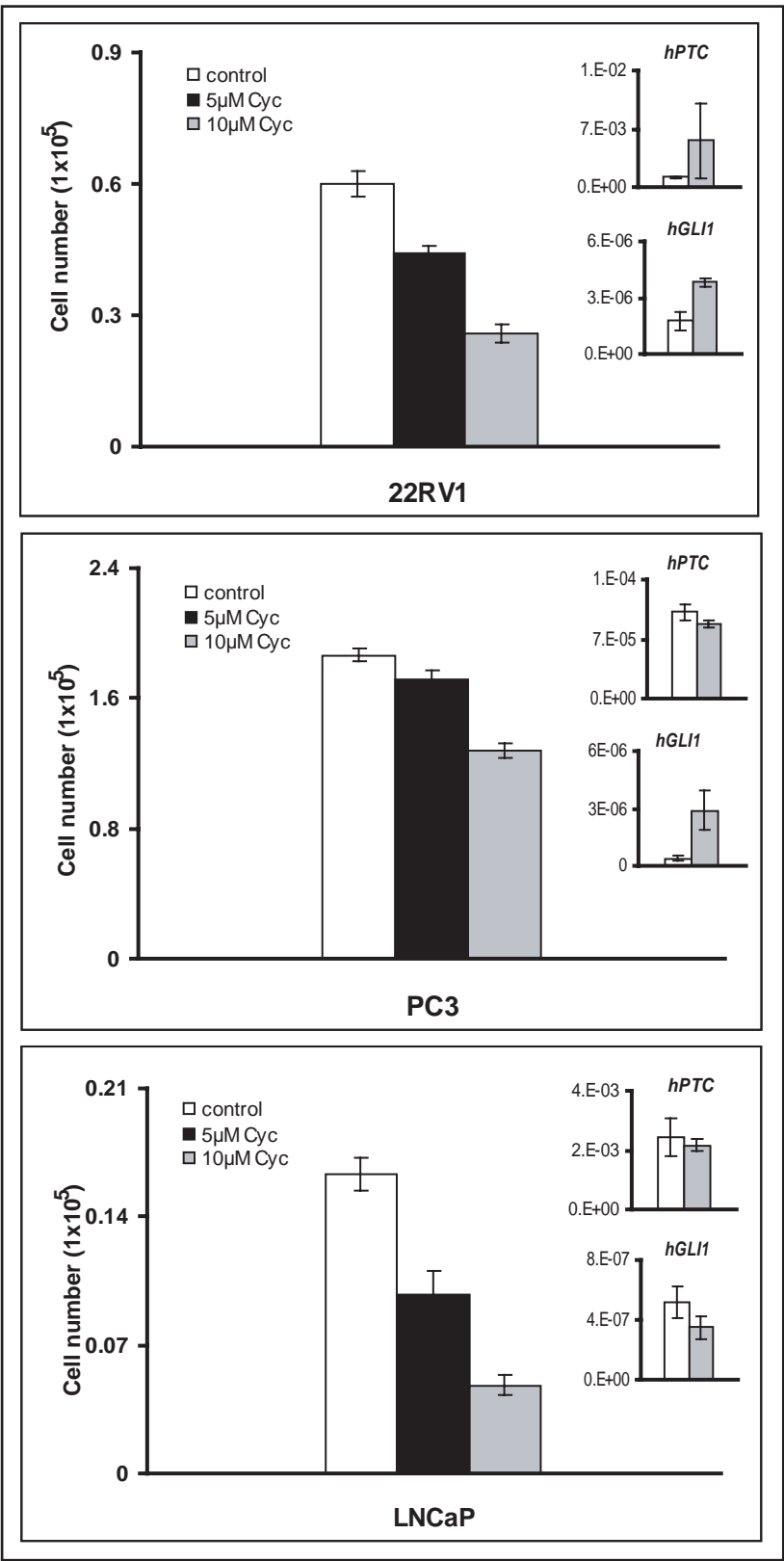
Figure 7 Proliferation of 22RV1, PC3, and LNCaP cells over 4 days was inhibited by cyclopamine in a dose dependent fashion ($p<0.05$ at 10uM cyclopamine). In these cultures expression of the Hh target genes Ptc and Gli1 were not altered by 10uM cyclopamine suggesting that the reduction in proliferation was not through a Smo-mediated event (insert).
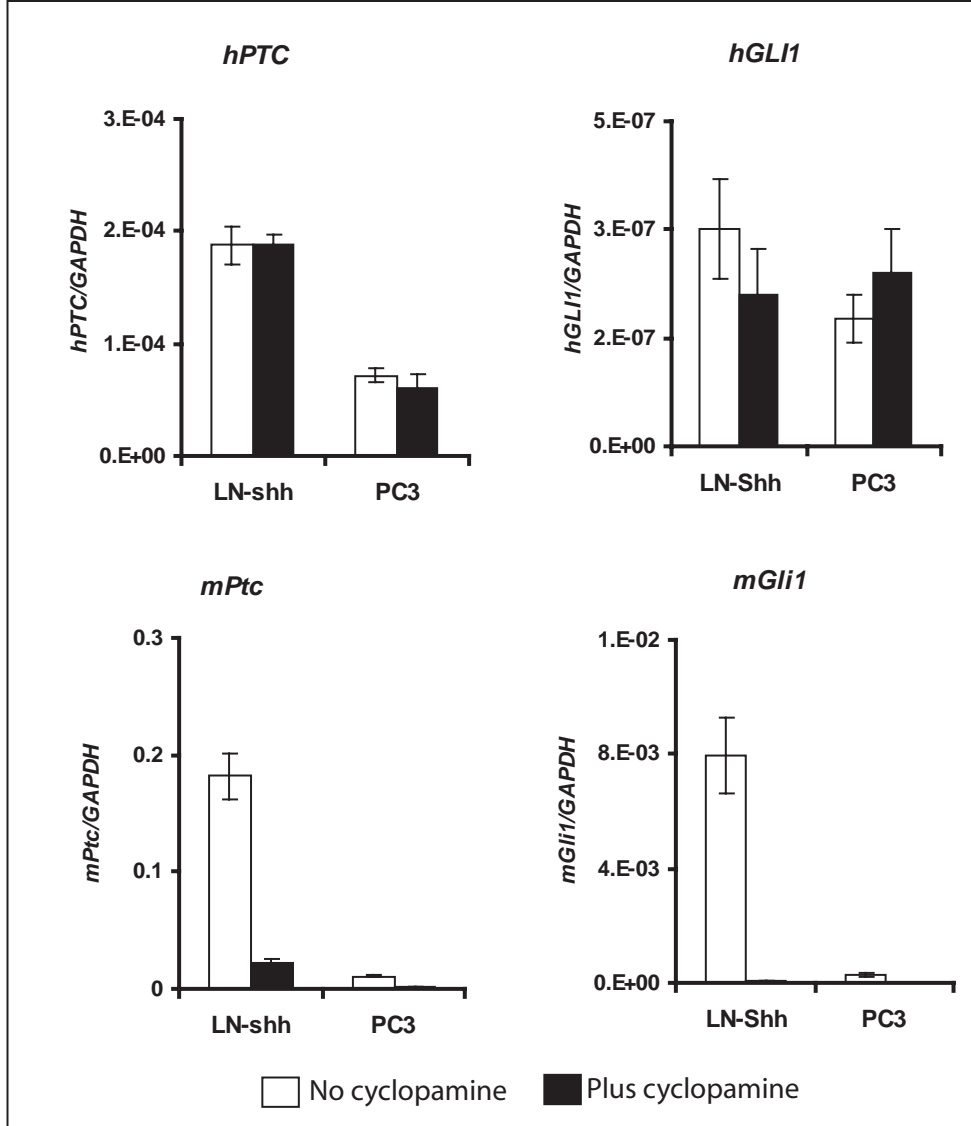
Figure 8 Effect of 10uM cyclopamine on autocrine and paracrine pathway activity in co-cultures of either LNCaP cells over-expressing Shh (LN-Shh) or PC3 cells co-cultured with mouse UGSM-2 cells. There is no effect on expression of hPtc and hGli1 (upper panel). However inhibition of paracrine signaling in UGSM-2 co-cultured with either LN-Shh or PC3 is evident from the decrease in mPtc and mGli1 expression in the presence of cyclopamine (lower panel; $p < 0.05$).

HEDGEHOG SIGNALING IN THE PROSTATE

AUBIE SHAW AND WADE BUSHMAN*

From the McArdle Laboratory for Cancer Research and the Department of Surgery,
University of Wisconsin, Madison, Wisconsin

*Please send correspondence to: Department of Surgery, University of Wisconsin, 600
Highland Avenue, Madison, WI 53792 (telephone: 608-265-8705, FAX: 608-265-8133,
e-mail: bushman@surgery.wisc.edu).

Running title: Hh signaling in prostate

Key words: hedgehog pathway, prostate development, prostate cancer, tissue stem cells,
inflammation

Abbreviations: Prostate cancer; PCa, Hedgehog; Hh, Patched; Ptc, Sonic Hedgehog; Shh,
Indian Hedgehog; Ihh, Smoothened; Smo, Hedgehog-Interacting Protein ; Hip,
Suppressor of Fused; SuFu

Word count: 4047

ABSTRACT

Purpose: Recent discoveries have highlighted the importance of the Hedgehog (Hh) signaling pathway in prostate growth regulation. This paper reviews the role of Hh signaling in prostate development, adult prostate homeostasis and prostate cancer. Materials and Methods: A comprehensive review of all relevant literature was conducted. Results: Epithelial expression of Hh ligand during prostate development exerts both autocrine and paracrine signaling activities that regulate growth and differentiation. Hh signaling also occurs in the adult human prostate but the influence on epithelial proliferation and/or differentiation is unknown. Robust Hh signaling occurs frequently in prostate cancer, and both autocrine and paracrine signaling have been shown to accelerate the growth of xenograft tumors. Autocrine signaling has been implicated in stimulating stem/progenitor cells and increased Hh pathway activity may be a characteristic of advanced, androgen independent cancer. The plant alkaloid cyclopamine is a specific chemical inhibitor of Hh signaling and has produced sustained regression of established xenograft tumors.
Conclusions. Hh signaling plays an important role in prostate development and appears to be a characteristic feature of prostate cancer. It stimulates tumor growth and may exert a specific role in the proliferation of tumor stem cells. The development of Hh inhibitors based on the action of cyclopamine holds promise for novel treatments to slow or arrest tumor growth.

WHAT'S SO EXCITING ABOUT HEDGEHOG?

In a recent editorial comment in this journal, Patrick Walsh described recent findings regarding the role of Hh signaling in PCa as being among the most important basic science findings related to PCa in the past 30 years[1]. Hh was first identified as an important signaling molecule in *Drosophila*. Hh signaling is conserved in vertebrates and plays an important role in fetal development of diverse structures including the prostate gland. Recent work has shown that Hh signaling promotes PCa growth and activated Hh signaling has been identified as a key feature of clinically advanced disease. Even more exciting is the possible connection of Hh signaling to proliferation of tumor stem cells, the small compartment of cells within a tumor that may be responsible for androgen-independent tumor recurrence. Specific chemical inhibitors of Hh signaling have produced sustained regression of various xenograft tumors without overt toxicity to the adult host, suggesting that they may represent an entirely new class of therapeutic agents that could target previously untreatable cancers.

RELEVANCE OF DEVELOPMENTAL STUDIES TO CANCER

The Hh transcriptional activator Gli1 was first identified as an oncogene in glioblastoma[2]. Inactivating mutations in the Hh receptor Ptc were found in medulloblastomas[3] and Gorlin/nevoid basal cell carcinoma syndrome[4, 5]. More recently, aberrant Hh signaling has been found to be a consistent feature of a variety of tumors originating in organs where Hh signaling plays an important developmental role, including sporadic basal cell carcinoma of skin, pancreatic cancer, small cell lung cancer, gastric cancer and PCa, prompting widespread speculation that reactivation of developmental signaling pathways is a critical step in tumor development.

A STEM CELL CONNECTION

An important facet of Hh signaling is its connection to stem cell proliferation[6]. Recent studies have shown a role for Hh signaling in stem/progenitor cell proliferation in the CNS, mammary gland[7], skin[8], gut[9] and pancreas[10, 11]. Hh signaling localizes to germinal cell populations in the developing CNS and is required for maintenance and expansion of progenitors[12, 13]. Disruption of Hh signaling in the fetal brain reduces the number of neural progenitors while Hh pathway activation in the mature brain increases proliferation of telencephalic progenitors[12] and sustained pathway activation produces medulloblastomas[14, 15]. These findings have ignited speculation that Hh signaling is a key factor in sustaining proliferation of tumor stem cells.

A THERAPEUTIC OPPORTUNITY

Craniofacial birth defects in lambs born in Idaho in the 1950's were ultimately traced to the teratogenic effects of an alkaloid, cyclopamine, in the plant *Veratrum californicum.* The similarity to defects observed in the *Shh* null mouse[16] led to the discovery that cyclopamine is a specific chemical inhibitor of Hh signal transduction[17]. Cyclopamine has been used to examine the effect of Hh pathway inhibition on tumor growth and has shown dramatic treatment efficacy in animal models of basal cell carcinoma, medulloblastoma, pancreatic cancer and PCa. Recently, topically applied cyclopamine showed remarkable efficacy against basal cell carcinoma of skin in humans[18].

OVERVIEW OF HH SIGNALING

Of the three mammalian Hh genes Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh), Shh is the most widely expressed during development. Shh binds to a specific receptor Ptc on the target cell surface and activates an intracellular signal transduction pathway involving the Gli family of transcription factors that activates transcription of specific genes in the target cell (summarized in Figure 1).

Hh signaling is regulated at several levels. The transmembrane Ptc receptor constitutively represses Hh pathway activity through its interaction with a second transmembrane protein, Smo. Binding of Hh ligand to Ptc disrupts this interaction and de-represses pathway activity. Induction of Ptc expression by Hh signaling creates a negative feedback loop that re-asserts repression at the level of the membrane. A second mechanism for negative feedback is provided by Hh-induced expression of Hip, a cell surface glycoprotein that sequesters Hh ligand. Three Gli genes (Gli1, Gli2, and Gli3) encode transcriptional regulators which share a conserved DNA-binding domain and bind the same 9bp recognition sequence. Gli1 is a transcriptional activator of Hh target genes. Gli2 provides redundancy in the transcriptional activating functions of Gli1. Gli3 functions primarily as a transcriptional repressor that balances and refines transcriptional activation by Gli1 and Gli2. A third domain of Hh pathway regulation depends upon a complex network of regulatory elements in the cytoplasm, involving PKA and several other proteins including Fused (Fu), Supressor of Fused (SuFu) and Costal 2 (Cos2) which regulate the location and activity of Gli proteins[19].

HH SIGNALING IN PROSTATE DEVELOPMENT

During prostate ductal morphogenesis, Shh expression localizes to sites of active growth. During ductal budding, Shh expression in the epithelium is up-regulated and condenses at sites of epithelial evagination. During ductal outgrowth, Shh expression is strongest at the duct tip. Shh expression in the urogenital sinus (UGS) is not dependent upon testosterone, but testosterone does modestly increase the level of expression and Shh redistribution during budding is certainly tied to an androgen-induced morphogenetic event[20]. Blockade of Hh signaling by antibody blockade or chemical inhibition of Hh signaling disrupts ductal budding and glandular morphogenesis, respectively[20, 21]. However, Berman et al[22] and Freestone et al[23] both observed budding of the Shh transgenic null UGS and glandular morphogenesis in subcapsular renal grafts. The apparent discrepancy between these observations was resolved by our recent finding that Ihh provides functional redundancy for Shh. Impairment of Hh signaling by transgenic Gli2 loss of function results in decreased Hh target gene expression, disruption of ductal budding, diminished expression of the stem cell marker Nestin and hyperplasia of p63$^+$ basal cells[24]. These studies show that Hh signaling and Gli-mediated transactivation of Hh target genes is required for normal ductal budding, and to balance progenitor cell proliferation and differentiation.

Hh signaling can occur between tissue layers (paracrine signaling) or among cells within the same tissue layer (autocrine signaling). Ptc and Gli1, targets of Hh signaling, are tightly localized in the mesenchyme surrounding the nascent buds of the developing prostate. Localization of Shh expression to the tip of the elongating ducts is mirrored by Gli1 and Ptc expression in the surrounding mesenchyme[20, 25]. Paracrine signaling directly affects mesenchymal proliferation[23, 26] but also influences epithelial proliferation and differentiation by paracrine feedback mechanisms[20, 26-28]. In addition, there is concentrated epithelial expression of Ptc and Gli1, an indication of autocrine signaling, in the nascent buds and at the tips of the growing ducts[25,29]. Given that autocrine signaling stimulates progenitor cell proliferation in other organs, it is tempting to speculate that autocrine signaling at the tips of growing buds plays a role in progenitor epithelial cell expansion (Figure 2).

Several recent observations are consistent with a role for Hh signaling in the maintenance and/or proliferation of prostatic progenitor cells. Gli2 loss of function and impaired Hh signaling are associated with decreased expression of the stem cell marker Nestin[24] in the prostate. Castration-induced regression of the ventral prostate is associated with increased expression of Hh ligand, Smo, Gli1 (indicating increased Hh signaling) and this is paralleled by increased Nestin expression. These changes are all reversed during testosterone induced re-growth (unpublished observations) and, remarkably, chemical blockade of Hh signaling prevents testosterone-induced re-growth[30].

INFLAMMATION IN PROSTATE CANCER

Chronic inflammation and oxidative stress have been identified as key factors in predisposing to the development of PCa[31] and, indeed, lesions in the human prostate characterized by proliferating epithelial cells and activated inflammatory cells (proliferative inflammatory atrophy) are considered likely precursors of PIN and PCa[32-34]. An emerging paradigm postulates that epithelial injury and inflammation activates

proliferation of stem cells as part of the repair process. These proliferating progenitor cells are exposed to oncogenic forces such as oxidative stress that can induce genetic or epigenetic changes leading to a persistent state of activation. The interaction between the persistently activated progenitor cell and the reactive stroma associated with inflammation and healing results in tumor formation and unregulated growth. The Hh and Wnt signaling pathways have been identified as the two critical pathways regulating stem cell activation. In some tissues, such as the colon, activated Wnt signaling appears to be the dominant actor. In other tissues, such as the brain, skin, lung, pancreas and prostate, Hh signaling appears to play a key role in regulating stem cell activation and tumor development[6].

HH SIGNALING IN THE DEVELOPING AND ADULT PROSTATE

Shh is abundantly expressed in the human fetal prostate and is down-regulated before birth[27, 35]. A highly quantitative analysis of Hh signaling in the adult prostate by Fan et al[27] utilized real-time RT-PCR to compare Shh, Gli1, Gli2 and Gli3 expression in normal prostate tissue from organ donors, BPH tissue obtained by prostatectomy, and both tumor and zone-homologous normal tissue from radical prostatectomy specimens. The human fetal brain and fetal prostate were included as reference controls. These studies showed that expression of Shh and Gli1 in specimens of normal prostate and BPH varied over several orders of magnitude but was generally comparable to the robust level of expression observed in the fetal brain. A tight correlation between Shh and Gli1 expression was observed, consistent with a dependence of Gli1 expression on Shh signaling. Karhadkar and colleagues[30] did not examine expression in the normal prostate per se, but performed RT-PCR analysis for presence or absence of Shh, Ihh and pathway gene expression in primary epithelial (PrE) cells, benign prostate tissue adjacent to tumors, localized PCa, and PCa metastases. In their assays, they observed that Shh and Ihh were expressed in PrE cells, tumor associated benign tissue and localized PCa, but the conserved Hh target genes Ptc and Gli1 were not. Ptc and Gli1 were only expressed in metastatic tumors. Sanchez et al[36] examined expression in normal human prostate tissue using real-time RT-PCR to compare expression of Shh, Ptc and the Gli genes in six specimens of human PCa and tumor associated benign tissue. These studies, combined with immunostaining of a tissue microarray containing both tumor and tumor-associated benign tissue, suggested a basal level of Shh, Ptc and Gli1 expression in the benign tissue that is variably increased in tumor. Neither the Sanchez nor Karhadkar studies included a reference control such as fetal brain to establish the relative level of expression in their specimens. This led to the widely shared perception that the level of expression of Shh and Ptc and Gli1 is "low" in benign prostate tissue, but this interpretation is incorrect. The quantitative comparisons provided by Fan et al clearly show that expression of Shh and Gli1 in normal adult and benign prostate tissues rivals the robust level of expression seen in the fetal brain. This is reinforced by a recent comparison of expression in a pooled specimen of 30 normal prostate tissues and the fetal brain, showing high levels of expression of Shh, Ptc, Gli1 and Smo in the normal prostate[37].

In situ hybridization studies using a highly specific radiolabeled probe localized Shh expression to the prostatic epithelium and Gli1 expression almost exclusively to the periglandular stroma. Ptc, which is expressed at a basal level in the absence of Hh

pathway activity, was expressed in both compartments[27]. Sanchez et al[36] performed in situ hybridization with a digoxigenin-labeled probe and immunostaining to demonstrate relatively weak co-expression of Shh, Ptc and Gli1 in the prostatic epithelium. These studies suggest that Hh signaling in the normal/benign adult prostate may involve a combination of autocrine and paracrine signaling. While the role of Hh signaling in the adult prostate are not yet known, studies of Hh signaling during prostate development suggest a diverse repertoire of potential activities. Studies of Hh signaling in early prostate development highlighted a role for Hh signaling in stimulating epithelial proliferation. In contrast, studies examining the effect of Hh signaling in the postnatal prostate suggested that Hh signaling inhibits proliferation and stimulates terminal epithelial differentiation. These studies make clear that Hh signaling exerts multiple effects, both growth stimulatory and growth inhibitory[20, 22, 23, 25, 26, 29]. These activities may be distinguished by autocrine versus paracrine signaling mechanisms and/or by an evolving response of the mesenchyme to paracrine signaling as it differentiates. Whatever the case, it is clear that Hh signaling evokes a variety of effects that might underpin homeostatic growth regulation in the normal adult prostate as well as in response to epithelial injury and inflammation.

HH SIGNALING IN PROSTATE CANCER

Studies of Hh signaling in human PCa suggest that (1) both autocrine and paracrine signaling contribute to tumor growth, (2) the effect of paracrine signaling may be influenced by the reactive character of the tumor stroma, and (3) ligand dependent and ligand independent autocrine pathway activation is a feature of advanced disease. Fan et al[27] compared Shh and Gli gene expression in tumor specimens obtained by radical prostatectomy to expression in specimens of BPH and normal prostate. Mean expression in tumors was nearly an order of magnitude higher than in the benign specimens, though the difference was not statistically significant because of the wide range of expression in benign specimens. In a separate analysis, tumor and zone matched benign tissue from the same patients was examined and this showed generally comparable levels of robust Shh expression in both tissues from the same patient. Karhadkar et al[30] used RT-PCR analysis to compare Hh ligand expression and Hh pathway activity in specimens of localized and metastatic PCa. They showed that Hh ligand was expressed abundantly in both localized and metastatic PCa, but that Hh pathway activity, as evidenced by Ptc and Gli1 expression, was dramatically increased in metastatic lesions. They attributed this to an increased responsiveness to Hh ligand conferred by renewed expression of Smo. An alternative explanation is that the Hh response in metastases is due to increased Hh sensitivity of stroma at metastatic sites. Sheng[38] also demonstrated an increase in Ptc expression in advanced PCa, and attributed some of the increase to mutations in SuFu leading to dysregulated autocrine pathway activity. Sanchez[36] used RT-PCR analysis to demonstrate a variable increase in Shh expression and pathway activity in tumor tissue as compared to matched benign tissue from the same specimen and used immunostaining for Shh to show that increased Shh expression occurred in nearly 33% of tumor specimens as compared to <1% in benign tissues. Together, these studies suggest that high levels of Shh and Gli1 expression is found in localized prostate tumors as well as benign, zone homologous tissue in the same gland and that a further increase in Shh expression and Hh signaling occurs in advanced PCa.

Localization studies performed by Fan et al[27] showed Shh expression in the tumor and glandular epithelium and Gli1 expression primarily in the periductal stroma. Sanchez et al[36] performed both in situ hybridization and immunostaining and showed Shh, Ptc and Gli1 expression co-localizing to the tumor epithelium. The apparent discrepancy in the location of Gli1 expression - and therefore the cell type exhibiting pathway activation - could be a product of different methods of assay and/or might reflect heterogeneity of autocrine and paracrine signaling in PCa.

The commonly used PCa cell lines LNCaP, PC3, 22RV1 and DU145 all express Shh and Ihh as well as the major components of the Hh pathway. The levels of expression vary considerably and the secretion of functional ligand has not been confirmed in most cases. Work presented in three different papers has suggested that autocrine signaling in tumor cell lines stimulates cell proliferation, however, there are significant discrepancies in the findings in different laboratories. Karhadkar et al[30] found that anti-Shh blocking antibody inhibited PC3 proliferation in culture, suggesting that ligand-dependent autocrine signaling stimulates cell proliferation. However, Sanchez et al[36] found that PC3 proliferation was unaffected by either anti-Shh blocking antibody or recombinant Shh. The discrepancy in these results has not been resolved. The Hh pathway inhibitor cyclopamine was found to inhibit proliferation of PC3 and LNCaP cells in culture[30, 36, 38]. Cyclopamine inhibited Gli1 expression in LNCaP cells, arguing that the effect is pathway specific, however, the unresponsiveness of LNCaP cells to exogenous Shh[36] argues against operation of a ligand-dependent pathway. The potential of chemical blockade of Hh signaling to inhibit tumor growth was examined by administering cyclopamine to mice with human PCa xenografts[30]. Both PC3 and 22RV1 tumors showed a dose dependent inhibition of tumor growth, and complete and sustained regression at the highest dose tested. The specificity of this effect was confirmed by showing that xenografts made with tumor cells overexpressing Gli1 were resistant to the anti-tumor effect of cyclopamine. These studies were interpreted as evidence that autocrine signaling in the PC3 and 22RV1 tumors promotes tumor growth and can be inhibited by cyclopamine blockade. Additional experiments performed with rodent tumor cell lines showed that cyclopamine could inhibit growth and metastasis of the aggressive AT6.3 cell line and that Gli1 overexpression conferred a highly aggressive and metastatic phenotype to the normally less aggressive AT2.1 cell line. While these observations are all consistent with the notion that cyclopamine inhibited tumor growth by blocking Hh signaling, it is important to point out that the effect of cyclopamine on growth of PC3, 22RV1 and AT6.3 tumors was not correlated with an inhibition of Hh signaling. Detailed studies under a variety of conditions in our laboratory showed that LNCaP, PC3 and 22RV1 do not exhibit the canonical transcriptional response to Hh ligand. In addition, cyclopamine treatment did not produce an inhibition of Ptc and Gli1 expression even at concentrations that inhibited cell growth in culture. These observations, clearly at odds with previously published observations, were complemented by transfection-based studies showing that the Hh signal transduction pathway is non-functional in both PC3 and 22RV1. These findings are important for three reasons. First, they show that PC3 and 22RV1 cannot be used to model ligand-dependent autocrine signaling in human PCa. Second, they demonstrate that expression of Ptc and Gli1 in PC3 and 22RV1 is

independent of the canonical Hh signal transduction mechanism and therefore may be an inappropriate model for studying ligand-independent pathway activation that results from dysregulation of signal transduction. Third, these cell lines are not appropriate models for testing Hh pathway inhibitors based on cyclopamine's mechanism of action.

The effect of paracrine signaling on tumor growth was examined using the LNCaP xenograft. Overexpression of Shh by LNCaP tumor cells increased expression of Ptc and Gli1 in the tumor stroma, without any evidence of autocrine pathway activation, and accelerated tumor growth[27]. This suggested that Shh expressed by the tumor cells acted on adjacent stromal cells to elicit paracrine signals that promoted tumor growth. Recently, we have shown that Hh pathway activation in the tumor stroma alone is sufficient to accelerate tumor growth (unpublished observations). Other recent studies show that the effect of tumor cell Shh expression on tumor growth is determined by the phenotype of the tumor stroma (unpublished observations). The dominant effect of the stromal phenotype on the growth response to paracrine signaling may explain the differing effects of Hh expression in the growth quiescent normal prostate and in prostate cancer where a reactive stroma is generally present[39] (Figure 3).

HH SIGNALING, ANGIOGENESIS AND METASTASIS

Vascular endothelium is a well established target of Hh signaling. Shh induces expression of pro-angiogenic molecules including vascular endothelial growth factors (VEGF) and angiopoietins (Agpt) by stromal cells. VEGF and Agpt stimulate endothelial proliferation and growth of vessels into tumors. Hedgehog-interacting protein, an inhibitor of Hh signaling, is abundantly expressed in resting endothelial cells and is downregulated in PCa xenografts undergoing angiogenesis[40]. The pro-angiogenic effects of Hh may provide a growth stimulus for tumors and also a means to metastasize.

Hh signaling correlates with metastatic potential and Gli1 overexpression can render a non-metastatic cell line metastatic (Karhadkar 2004). Hh signaling is implicated in mediating epithelial-mesenchymal transition (EMT), an event that is postulated to facilitate carcinoma invasion. Overexpression of Gli1 in a non-metastatic PCa cell line stimulated expression of Snail, a marker of EMT, to levels seen in metastatic lines and increased cell invasion in vitro. In addition, Hh signaling may contribute to the predilection of PCa for bony metastasis since bone marrow stromal cells are responsive to Hh ligands and both Shh and Ihh stimulate bone remodeling[41, 42].

CONCLUSIONS
Robust Hh signaling is characteristic of the adult human prostate and may play a variety of roles in homeostatic growth regulation and the response to injury or inflammation. Hh ligand expression and pathway activity is common in localized PCa and may promote tumor cell proliferation by a combination of autocrine and paracrine signaling. Some of this may occur by canonical ligand dependent mechanisms and some may involve, as suggested by Sheng et al[38], mutations affecting the regulation of Hh pathway activity in the tumor cells. Hh pathway activity is dramatically increased in advanced, metastatic PCa but whether this represents mutational activation or an increased responsiveness of the tumor cell or ectopic stroma to Hh ligand is not known.

Hh signaling is a unique target for therapy both because of the apparently limited toxicity associated with chemical inhibition and the potential of this pathway to attack the postulated stem cell core of PCa. Recognizing that success in animal xenograft studies frequently doesn't translate to success in treating human cancers, what can we realistically expect? The first point to make is that Hh signaling occupies a unique niche in the signaling realm. There is some level of functional redundancy at both the level of the ligand (Shh, Ihh, Dhh) and at the level of target gene regulation (Gli1, Gli2, Gli3), but the signal transduction pathway appears to funnel specifically through the Ptc/Smo complex at the membrane level. There is little known cross-talk involving the Ptc/Smo receptor and it is therefore likely that inhibitors targeted to Ptc/Smo will allow little room for escape by physiologic mechanisms. Paracrine or autocrine signaling which occurs by a ligand dependent mechanism are therefore promising targets for therapy. The stromal response involved in paracrine signaling is especially likely to depend upon the canonical ligand-dependent pathway and is therefore a prime target for therapy to slow or arrest tumor progression. The relative contributions ligand-dependent autocrine signaling and mutational activation of the pathway in localized and metastatic tumor growth is as yet unknown. Autocrine signaling that proceeds through an intact signal transduction pathway and regulatory mechanisms is likely to be responsive to Hh blockade by analogs of cyclopamine, however, autocrine pathway activity that occurs downstream from Ptc/Smo, through inactivation of SuFu for example, can be expected to escape the action of cyclopamine-like inhibitors. Thus, it is possible that as tumors progress and acquire an increasing number of mutations they could acquire changes that result in autocrine pathway activation that is un-responsive to the Hh inhibitors based on the action of cyclopamine.

What is needed? The overly simplistic conclusion that Hh signaling is increased in PCa and that tumor growth can be stopped by treatment with Hh inhibitors like cyclopamine needs to be refined. We now know that Hh signaling is present in the normal prostate as well as in cancer and in order to really understand what's going on, we have to understand how the roles of Hh signaling are similar and different in the normal prostate and in PCa. This will entail further studies to define the relative abundance of autocrine and paracrine signaling in the normal prostate, localized cancer and metastatic cancer and mechanistic studies to examine how these activities are related to stem cell proliferation, amplifying or transit cell proliferation/differentiation, and androgen regulation of growth and invasion. Moreover, we must identify what proportion of autocrine signaling in PCa is ligand dependent and what proportion results from intracellular pathway mutations. This information will enable us to select the tumor cell lines, xenograft models, and animal models that most accurately represent the human tumor and use these for drug development and testing. A novel and minimally toxic intervention that can cut to the root of a tumor is an exciting prospect for the treatment of PCa. Realization of the goal will require a great deal of work but it may not be so far away.

<div align="center">REFERENCES</div>

1.	Walsh, P. C.: Hedgehog signalling in prostate regeneration, neoplasia and metastasis. J Urol, **173:** 1169, 2005

2.	Kinzler, K. W., Bigner, S. H., Bigner, D. D. et al.: Identification of an amplified, highly expressed gene in a human glioma. Science, **236:** 70, 1987

3.	Raffel, C., Jenkins, R. B., Frederick, L. et al.: Sporadic medulloblastomas contain PTCH mutations. Cancer Res, **57:** 842, 1997

4.	Hahn, H., Wicking, C., Zaphiropoulous, P. G. et al.: Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. Cell, **85:** 841, 1996

5.	Johnson, R. L., Rothman, A. L., Xie, J. et al.: Human homolog of patched, a candidate gene for the basal cell nevus syndrome. Science, **272:** 1668, 1996

6.	Beachy, P. A., Karhadkar, S. S., Berman, D. M.: Tissue repair and stem cell renewal in carcinogenesis. Nature, **432:** 324, 2004

7.	Liu, B. Y., McDermott, S. P., Khwaja, S. S. et al.: The transforming activity of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells. Proc Natl Acad Sci U S A, **101:** 4158, 2004

8.	Hutchin, M. E., Kariapper, M. S., Grachtchouk, M. et al.: Sustained Hedgehog signaling is required for basal cell carcinoma proliferation and survival: conditional skin tumorigenesis recapitulates the hair growth cycle. Genes Dev, **19:** 214, 2005

9.	Ramalho-Santos, M., Melton, D. A., McMahon, A. P.: Hedgehog signals regulate multiple aspects of gastrointestinal development. Development, **127:** 2763, 2000

10.	Hebrok, M., Kim, S. K., St Jacques, B. et al.: Regulation of pancreas development by hedgehog signaling. Development, **127:** 4905, 2000

11.	Kawahira, H., Scheel, D. W., Smith, S. B. et al.: Hedgehog signaling regulates expansion of pancreatic epithelial cells. Dev Biol, **280:** 111, 2005

12.	Machold, R., Hayashi, S., Rutlin, M. et al.: Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. Neuron, **39:** 937, 2003

13.	Palma, V., Ruiz i Altaba, A.: Hedgehog-GLI signaling regulates the behavior of cells with stem cell properties in the developing neocortex. Development, **131:** 337, 2004

14. Dahmane, N., Sanchez, P., Gitton, Y. et al.: The Sonic Hedgehog-Gli pathway regulates dorsal brain growth and tumorigenesis. Development, **128:** 5201, 2001

15. Ruiz i Altaba, A., Stecca, B., Sanchez, P.: Hedgehog--Gli signaling in brain tumors: stem cells and paradevelopmental programs in cancer. Cancer Lett, **204:** 145, 2004

16. Chiang, C., Litingtung, Y., Lee, E. et al.: Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature, **383:** 407, 1996

17. Incardona, J. P., Gaffield, W., Kapur, R. P. et al.: The teratogenic Veratrum alkaloid cyclopamine inhibits sonic hedgehog signal transduction. Development, **125:** 3553, 1998

18. Tabs, S., Avci, O.: Induction of the differentiation and apoptosis of tumor cells in vivo with efficiency and selectivity. Eur J Dermatol, **14:** 96, 2004

19. Hooper, J. E., Scott, M. P.: Communicating with Hedgehogs. Nat Rev Mol Cell Biol, **6:** 306, 2005

20. Lamm, M. L., Catbagan, W. S., Laciak, R. J. et al.: Sonic hedgehog activates mesenchymal Gli1 expression during prostate ductal bud formation. Dev Biol, **249:** 349, 2002

21. Podlasek, C. A., Barnett, D. H., Clemens, J. Q. et al.: Prostate development requires Sonic hedgehog expressed by the urogenital sinus epithelium. Dev Biol, **209:** 28, 1999

22. Berman, D. M., Desai, N., Wang, X. et al.: Roles for Hedgehog signaling in androgen production and prostate ductal morphogenesis. Dev Biol, **267:** 387, 2004

23. Freestone, S. H., Marker, P., Grace, O. C. et al.: Sonic hedgehog regulates prostatic growth and epithelial differentiation. Dev Biol, **264:** 352, 2003

24. Doles, J. D., Shi, X., Bushman, W.: Functional Redundancy of Hedgehog Signaling Regulating Mouse Prostate Development. Dev Biol, **in press**, 2005

25. Pu, Y., Huang, L., Prins, G. S.: Sonic hedgehog-patched Gli signaling in the developing rat prostate gland: lobe-specific suppression by neonatal estrogens reduces ductal growth and branching. Dev Biol, **273:** 257, 2004

26. Wang, B. E., Shou, J., Ross, S. et al.: Inhibition of epithelial ductal

branching in the prostate by sonic hedgehog is indirectly mediated by stromal cells. J Biol Chem, **278:** 18506, 2003

27.   Fan, L., Pepicelli, C. V., Dibble, C. C. et al.: Hedgehog signaling promotes prostate xenograft tumor growth. Endocrinology, **145:** 3961, 2004

28.   Lipinski, R. J., Cook, C. H., Barnett, D. H. et al.: Sonic hedgehog signaling regulates the expression of insulin-like growth factor binding protein-6 during fetal prostate development. Dev Dyn, **233:** 829, 2005

29.   Gao, N., Ishii, K., Mirosevich, J. et al.: Forkhead box A1 regulates prostate ductal morphogenesis and promotes epithelial cell maturation. Development, **132:** 3431, 2005

30.   Karhadkar, S. S., Bova, G. S., Abdallah, N. et al.: Hedgehog signalling in prostate regeneration, neoplasia and metastasis. Nature, **431:** 707, 2004

31.   Bostwick, D. G., Burke, H. B., Djakiew, D. et al.: Human prostate cancer risk factors. Cancer, **101:** 2371, 2004

32.   De Marzo, A. M., Meeker, A. K., Zha, S. et al.: Human prostate cancer precursors and pathobiology. Urology, **62:** 55, 2003

33.   Nelson, W. G., De Marzo, A. M., DeWeese, T. L. et al.: The role of inflammation in the pathogenesis of prostate cancer. J Urol, **172:** S6, 2004

34.   Palapattu, G. S., Sutcliffe, S., Bastian, P. J. et al.: Prostate carcinogenesis and inflammation: emerging insights. Carcinogenesis, **26:** 1170, 2005

35.   Barnett, D. H., Huang, H. Y., Wu, X. R. et al.: The human prostate expresses sonic hedgehog during fetal development. J Urol, **168:** 2206, 2002

36.   Sanchez, P., Hernandez, A. M., Stecca, B. et al.: Inhibition of prostate cancer proliferation by interference with SONIC HEDGEHOG-GLI1 signaling. Proc Natl Acad Sci U S A, **101:** 12561, 2004

37.   Zhang, J. X., Shaw, A., Lipinski, R. et al.: Autocrine/Paracrine Hedgehog Signaling and Ligand Dependence in Human Prostate Cancer Cell Lines. J Urol, **submitted**, 2005

38.   Sheng, T., Li, C., Zhang, X. et al.: Activation of the hedgehog pathway in advanced prostate cancer. Mol Cancer, **3:** 29, 2004

39.   Rowley, D. R.: What might a stromal response mean to prostate cancer progression? Cancer Metastasis Rev, **17:** 411, 1998

40.   Olsen, C. L., Hsu, P. P., Glienke, J. et al.: Hedgehog-interacting protein is highly expressed in endothelial cells but down-regulated during angiogenesis and in several human tumors. BMC Cancer, **4:** 43, 2004

41.   Edwards, P. C., Ruggiero, S., Fantasia, J. et al.: Sonic hedgehog gene-enhanced tissue engineering for bone regeneration. Gene Ther, **12:** 75, 2005

42.   St-Jacques, B., Hammerschmidt, M., McMahon, A. P.: Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes Dev, **13:** 2072, 1999
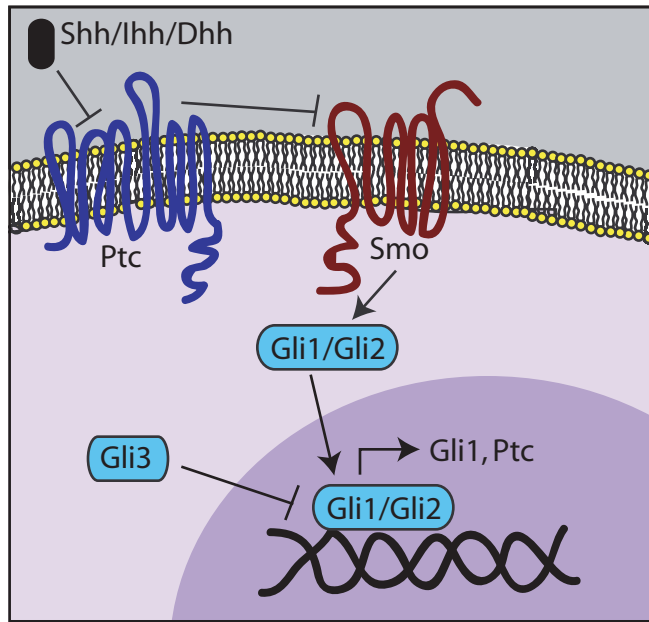
Figure 1. The mammalian Hh signaling pathway. Hedgehog ligands
Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh)
bind to the transmembrane receptor Patched (Ptc) and relieve constitutive repression of Smooth-
ened (Smo). Smo activation curtails transcriptional repression by Gli3 and promotes
activation/translocation of Gli1 and Gli2 to the nucleus, resulting in transcriptional activation of
Hedgehog target genes. Gli1 and Ptc are primary targets of Hh pathway activation and serve as
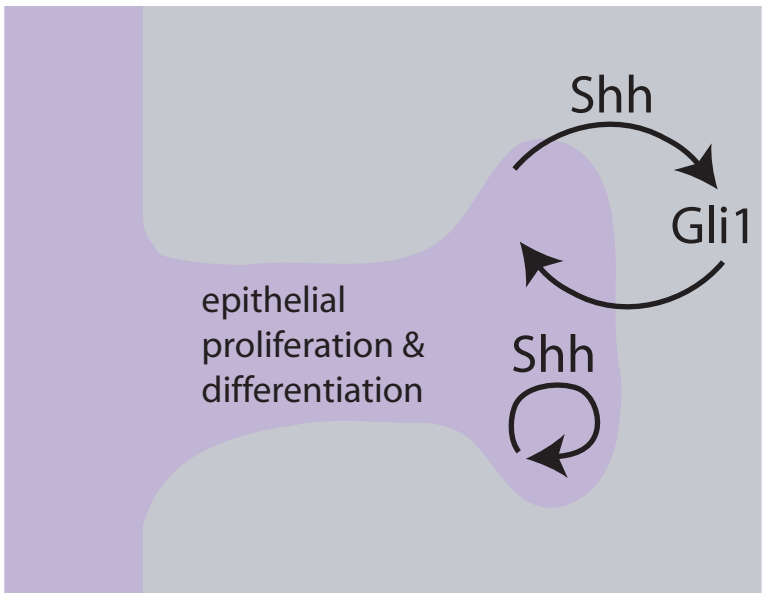reliable indicators of Hh signaling.
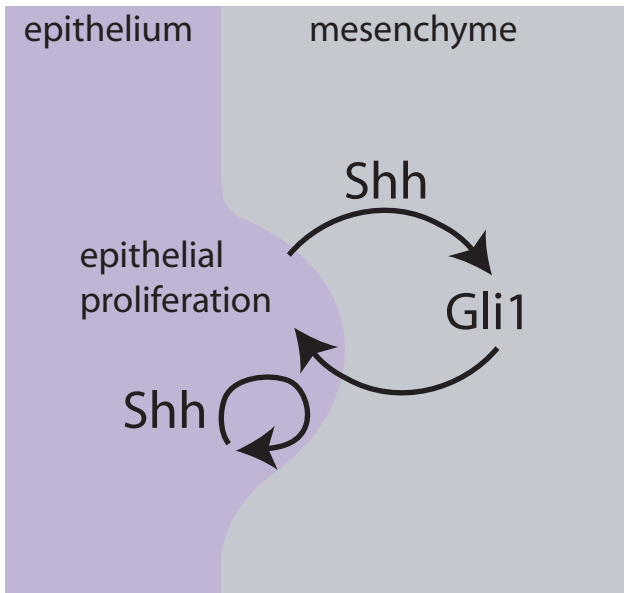
Figure 2. Postulated actions of Hh in prostate development. (Left) Androgen-dependent ductal budding is associated with focal expression of Shh in the epithelium of nascent buds. Shh acts on adjacent mesenchyme to activate expression of Hh target genes and increases epithelial proliferation (paracrine signaling). Autocrine signaling at the tip of the bud may stimulate progenitor cell proliferation. (Right) During ductal morphogenesis, autocrine signaling at the duct tip stimulates continued progenitor cell proliferation while paracrine signaling regulates epithelial differentiation.
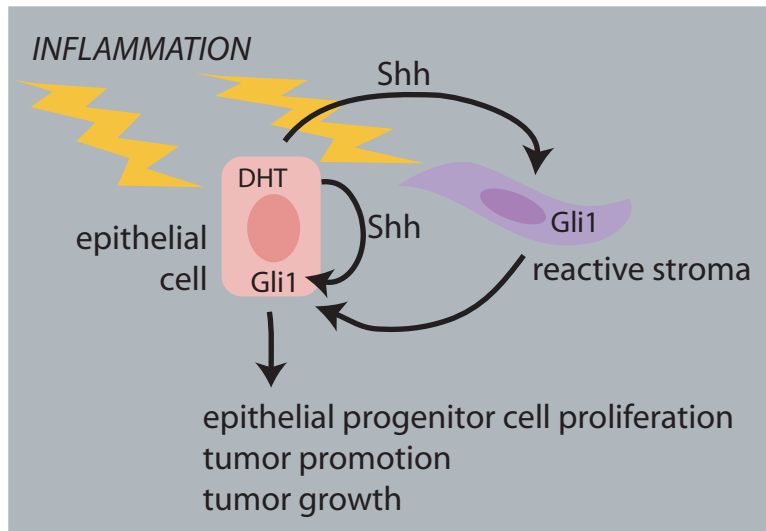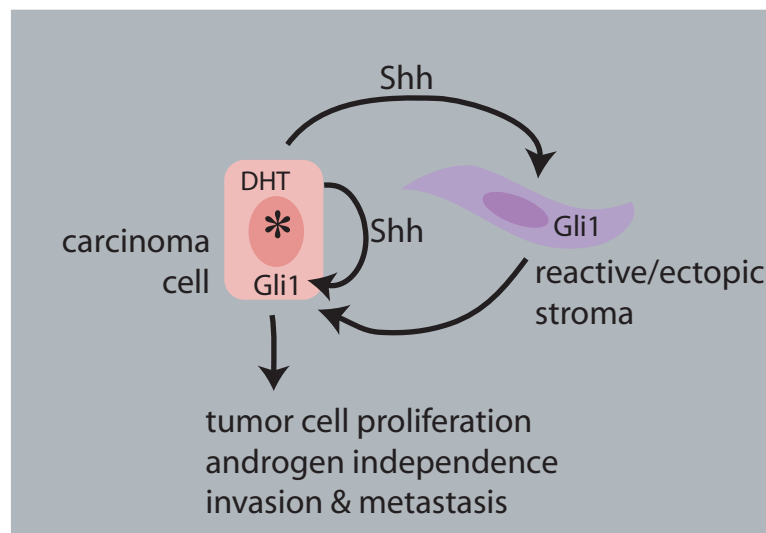
Figure 3. Postulated actions of Hh in prostate cancer. (Top) Injury and inflammation induce ligand-dependent autocrine stem cell activation and proliferation while paracrine signaling elicits growth stimulating responses from the reactive stroma. This creates an environment that promotes tumor formation and growth. (Bottom) Tumor growth is accelerated by ligand-dependent autocrine and paracrine signaling mechanisms and by mutations (*) that produce ligand-independent pathway activation. These activities promote invasion, metastasis and androgen independent tumor growth.